REGULATION OF HUMAN BMP-2 INDUCIBLE KINASE

This application incorporates by reference co-pending provisional application Serial
No. 60/367,512 filed March 27, 2002.

FIELD OF THE INVENTION

The invention relates to the regulation of human BMP-2 inducible kinase.

BACKGROUND OF THE INVENTION

Serine/threonine kinases, such as BMP-2 inducible kinase, catalyze the following
reaction: ATP + Protein serine/threonine = ADP + Protein serine/threonine
phospha$\because$. Because of the importance of this reaction, there is a need in the art to
identify additional serine/threonine kinases, which can be regulated to provide
therapeutic effects.

It is an object of the invention to provide reagents and methods of regulating a human
BMP-2 inducible kinase. This and other objects of the invention are provided by one
or more of the embodiments described below.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the DNA-sequence encoding a BMP-2 inducible kinase Polypeptide
(SEQ ID NO: 1).

Fig. 2 shows the amino acid sequence deduced from the DNA-sequence of Fig.1
(SEQ ID NO: 2).

Fig. 3 shows the DNA-sequence encoding a BMP-2 inducible kinase Polypeptide
(SEQ ID NO: 3).

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Fig. 4 shows the amino acid sequence deduced from the DNA sequence of Fig. 3
(SEQ ID NO: 4).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to an isolated polynucleotide from the group consisting of:

a)   a polynucleotide encoding a BMP-2 inducible kinase polypeptide comprising
an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 72% identical to
the amino acid sequence shown in SEQ ID NO: 2;
the amino acid sequence shown in SEQ ID NO: 2;

amino acid sequences which are at least about 72% identical to
the amino acid sequence shown in SEQ ID NO: 4; and
the amino acid sequence shown in SEQ ID NO: 4.

b)   a polynucleotide comprising the sequence of SEQ ID NOS: 1 or 3;

c)   a polynucleotide which hybridizes under stringent conditions to a poly-
nucleotide specified in (a) and (b) and encodes a BMP-2 inducible kinase
polypeptide;

d)   a polynucleotide the sequence of which deviates from the polynucleotide
sequences specified in (a) to (c) due to the degeneration of the genetic code
and encodes a BMP-2 inducible kinase polypeptide; and

e)   a polynucleotide which represents a fragment, derivative or allelic variation of
a polynucleotide sequence specified in (a) to (d) and encodes a BMP-2
inducible kinase polypeptide.

Human BMP-2 inducible kinase comprises the amino acid sequence shown in SEQ ID NO: 2. A coding sequence for human BMP-2 inducible kinase is shown in SEQ ID NO: 1. This sequence is contained within the longer sequence shown in SEQ ID

5   NO: 3. This sequence is located on chromosome 4q21.21. The 3D structure clearly shows that SEQ ID NO: 2 is an extracellular regulated kinase 2. A protein kinase domain is found from residues 176 to 189. Human BMP-2 inducible kinase of the invention is expected to be useful for the same purposes as previously identified BMP-2 inducible kinase enzymes. Furthermore, human BMP-2 inducible kinase can

10   be used in therapeutic methods to treat disorders such as cancer, diabetes, CNS disorders, COPD, gastrointestinal disorders or cardiovascular disorders. Human BMP-2 inducible kinase can also be used to screen for human BMP-2 inducible kinase activators and inhibitors.

15   One embodiment of the present invention is an expression vector containing any polynucleotide of the present invention.

Yet another embodiment of the present invention is a host cell containing any expression vector of the present invention.

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Still another embodiment of the present invention is a substantially purified BMP-2 inducible kinase polypeptide encoded by any polynucleotide of the present invention.

Even another embodiment of the present invention is a method of producing a BMP-

25   2 inducible kinase polypeptide of the present invention, wherein the method comprises the following steps:

a.   culturing the host cells of the present invention under conditions suitable for the expression of the BMP-2 inducible kinase polypeptide; and

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b.   recovering the BMP-2 inducible kinase polypeptide from the host cell culture.

Yet another embodiment of the present invention is a method for detecting a polynucleotide encoding a BMP-2 inducible kinase polypeptide in a biological sample comprising the following steps:

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    a.   hybridizing any polynucleotide of the present invention to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and

    b.   - detecting said hybridization complex.

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Still another embodiment of the present invention is a method for detecting a polynucleotide of the present invention or a BMP-2 inducible kinase polypeptide of the present invention comprising the steps of:

15   a.   contacting a biological sample with a reagent which specifically interacts with the polynucleotide or the BMP-2 inducible kinase polypeptide and

    b.   detecting the interaction

20   Even another embodiment of the present invention is a diagnostic kit for conducting any method of the present invention.

Yet another embodiment of the present invention is a method of screening for agents which decrease the activity of a BMP-2 inducible kinase, comprising the steps of:

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    a.   contacting a test compound with a BMP-2 inducible kinase polypeptide encoded by any polynucleotide of the present invention;

    b.   detecting binding of the test compound to the BMP-2 inducible kinase
30   polypeptide, wherein a test compound which binds to the polypeptide is

identified as a potential therapeutic agent for decreasing the activity of a BMP-2 inducible kinase.

Still another embodiment of the present invention is a method of screening for agents
5   which regulate the activity of a BMP-2 inducible kinase, comprising the steps of:

a.   contacting a test compound with a BMP-2 inducible kinase polypeptide encoded by any polynucleotide of the present invention; and

10   b.   detecting a BMP-2 inducible kinase activity of the polypeptide, wherein a test compound which increases the BMP-2 inducible kinase activity is identified as a potential therapeutic agent for increasing the activity of the. BMP-2 inducible kinase, and wherein a test compound which decreases the BMP-2 inducible kinase activity of the polypeptide is identified as a potential
15   therapeutic agent for decreasing the activity of the BMP-2 inducible kinase.

Even another embodiment of the present invention is a method of screening for agents which decrease the activity of a BMP-2 inducible kinase, comprising the step of:

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contacting a test compound with any polynucleotide of the present invention and detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent for decreasing the activity of BMP-2 inducible kinase.

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Yet another embodiment of the present invention is a method of reducing the activity of a BMP-2 inducible kinase, comprising the step of:

contacting a cell with a reagent which specifically binds to any polynucleotide of the
30   present invention or any BMP-2 inducible kinase polypeptide of the present invention, whereby the activity of BMP-2 inducible kinase is reduced.

Still another embodiment of the present invention is a reagent that modulates the activity of a BMP-2 inducible kinase polypeptide or a polynucleotide wherein said reagent is identified by any methods of the present invention.

Even another embodiment of the present invention is a pharmaceutical composition, comprising:

an expression vector of the present invention or a reagent of the present invention and a pharmaceutically acceptable carrier.

Yet another embodiment of the present invention is the use of an expression vector of the present invention or a reagent of the present invention for modulating the activity of a BMP-2 inducible kinase in a disease, preferably cancer, diabetes, CNS disorders, COPD, gastrointestinal disorders or cardiovascular disorders.

The invention thus provides a human BMP-2 inducible kinase that can be used to identify test compounds that may act, for example, as activators or inhibitors at the enzyme's active site. Human BMP-2 inducible kinase and fragments thereof also are useful in raising specific antibodies that can block the enzyme and effectively reduce its activity.

Polypeptides

Human BMP-2 inducible kinase polypeptides according to the invention comprise at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, or 1168 contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof, as defined below. A BMP-2 inducible kinase polypeptide of the invention therefore can be a portion of a BMP-2 inducible kinase protein, a full-length BMP-2 inducible

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kinase protein, or a fusion protein comprising all or a portion of a BMP-2 inducible kinase protein.

Biologically active variants

Human BMP-2 inducible kinase polypeptide variants which are biologically active, e.g., retain a kinase activity, also are human BMP-2 inducible kinase polypeptides. Preferably, naturally or non-naturally occurring human BMP-2 inducible kinase polypeptide variants have amino acid sequences which are at least about 72, preferably about 75, 80, 85, 90, 96, 96, or 98% identical to the amino acid sequence shown in SEQ ID NO: 2 or a fragment thereof. Percent identity between a putative human BMP-2 inducible kinase polypeptide variant and an amino acid sequence of SEQ ID NO: 2 is determined by conventional methods. See, for example, Altschul *et al., Bull. Math. Bio.* 48:603 (1986), and Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM62" scoring matrix of Henikoff & Henikoff, 1992.

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson & Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative variant. The FASTA algorithm is described by Pearson & Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444(1988), and by Pearson, *Meth. Enzymol.* 183:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NO: 2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed"

to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form

5   an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman & Wunsch, *J. Mol. Biol.48*:444 (1970); Sellers, *SIAM J. Appl. Math.26*:787 (1974)), which allows for amino acid insertions and deletions. Preferred parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap

10   extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol. 183*:63 (1990).

15   FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most preferably three, with other parameters set as default.

20   Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate

25   with a glutamate, or a threonine with a serine.

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing

30   biological or immunological activity of a human BMP-2 inducible kinase polypeptide

can be found using computer programs well known in the art, such as DNASTAR software.

The invention additionally, encompasses BMP-2 inducible kinase polypeptides that

5   are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications can be carried out by known techniques including, but not limited, to specific chemical cleavage by cyanogen

10   bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH$_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

Additional post-translational modifications encompassed by the invention include,

15   for example, e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of prokaryotic host cell expression. The BMP-2 inducible kinase polypeptides may also be

20   modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

The invention also provides chemically modified derivatives of BMP-2 inducible kinase polypeptides that may provide additional advantages such as increased

25   solubility, stability and circulating time of the polypeptide, or decreased immuno-genicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization can be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol, and the like. The polypeptides can be modified at random or predetermined

30   positions within the molecule and can include one, two, three, or more attached chemical moieties.

Whether an amino acid change or a polypeptide modification results in a biologically active BMP-2 inducible kinase polypeptide can readily be determined by assaying for enzymatic activity, as described for example, in Example 4.

Fusion proteins

Fusion proteins are useful for generating antibodies against BMP-2 inducible kinase polypeptide amino acid sequences and for use in various assay systems. For example, fusion proteins can be used to identify proteins that interact with portions of a human BMP-2 inducible kinase polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

A human BMP-2 inducible kinase polypeptide fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first polypeptide segment comprises at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, or 1168 contiguous amino acids of SEQ ID NO: 2 or of a biologically active variant, such as those described above. The first polypeptide segment also can comprise full-length BMP-2 inducible kinase protein.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include β-galactosidase, β-glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horse-radish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose-binding

protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the BMP-2 inducible kinase polypeptide-encoding sequence and the heterologous protein sequence, so that the BMP-2 inducible kinase polypeptide can be cleaved and purified away from the heterologous moiety.

A fusion protein can be synthesized chemically, as is known in the art. Preferably, a fusion protein is produced by covalently linking two polypeptide segments or by standard procedures in the art of molecular biology. Recombinant DNA methods can be used to prepare fusion proteins, for example, by making a DNA construct which comprises coding sequences selected from SEQ ID NO: 1 in proper reading frame with nucleotides encoding the second polypeptide segment and expressing the DNA construct in a host cell, as is known in the art. Many kits for constructing fusion proteins are available from companies such as Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), CLONTECH (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz, CA), MBL International Corporation (MIC; Watertown, MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

Identification of species homologs

Species homologs of human BMP-2 inducible kinase polypeptide can be obtained using BMP-2 inducible kinase polypeptide polynucleotides (described below) to make suitable probes or primers for screening cDNA expression libraries from other species, such as mice, monkeys, or yeast, identifying cDNAs which encode homologs of BMP-2 inducible kinase polypeptide, and expressing the cDNAs as is known in the art.

Polynucleotides

A human BMP-2 inducible kinase polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for a BMP-2 inducible kinase polypeptide. A coding sequence for human BMP-2 inducible kinase is shown in SEQ ID NO: 1.

Degenerate nucleotide sequences encoding human BMP-2 inducible kinase polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55, 60, 65, 70, preferably about 75, 90, 96, 98, or 99% identical to the nucleotide sequence shown in SEQ ID NO: 1 or its complement also are BMP-2 inducible kinase polynucleotides. Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of BMP-2 inducible kinase polynucleotides that encode biologically active BMP-2 inducible kinase polypeptides also are BMP-2 inducible kinase polynucleotides. Polynucleotide fragments comprising at least 8, 9, 10, 11, 12, 15, 20, or 25 contiguous nucleotides of SEQ ID NO: 1 or its complement also are BMP-2 inducible kinase polynucleotides. These fragments can be used, for example, as hybridization probes or as antisense oligonucleotides.

Identification of polynucleotide variants and homologs

Variants and homologs of the BMP-2 inducible kinase polynucleotides described above also are BMP-2 inducible kinase polynucleotides. Typically, homologous BMP-2 inducible kinase polynucleotide sequences can be identified by hybridization of candidate polynucleotides to known BMP-2 inducible kinase polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions--2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50 °C once, 30

minutes; then 2X SSC, room temperature twice, 10 minutes each--homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

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Species homologs of the BMP-2 inducible kinase polynucleotides disclosed herein also can be identified by making suitable probes or primers and screening cDNA expression libraries from other species, such as mice, monkeys, or yeast. Human variants of BMP-2 inducible kinase polynucleotides can be identified, for example,

10   by screening human cDNA expression libraries. It is well known that the T_m of a double-stranded DNA decreases by 1-1.5 °C with every 1% decrease in homology (Bonner et al., J. Mol. Biol. 81, 123 (1973). Variants of human BMP-2 inducible kinase polynucleotides or BMP-2 inducible kinase polynucleotides of other species can therefore be identified by hybridizing a putative homologous BMP-2 inducible

15   kinase polynucleotide with a polynucleotide having a nucleotide sequence of SEQ ID NO: 1 or the complement thereof to form a test hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly complementary nucleotide sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

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Nucleotide sequences which hybridize to BMP-2 inducible kinase polynucleotides or their complements following stringent hybridization and/or wash conditions also are BMP-2 inducible kinase polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook et al.,

25   MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50-9.51.

Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20 °C below the calculated T_m of the hybrid under study. The T_m of a hybrid between a BMP-2

30   inducible kinase polynucleotide having a nucleotide sequence shown in SEQ ID NO: 1 or the complement thereof and a polynucleotide sequence which is at least

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about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A. 48*, 1390 (1962):

5       $T_m = 81.5\,°C - 16.6(\log_{10}[Na^+]) + 0.41(\%G + C) - 0.63(\%formamide) - 600/l)$,

where l = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4X SSC at 65°C, or 50% formamide, 4X SSC at 42°C, or 0.5X SSC, 0.1% SDS at 65°C. Highly stringent wash conditions

10   include, for example, 0.2X SSC at 65°C.

Preparation of polynucleotides

A human BMP-2 inducible kinase polynucleotide can be isolated free of other cellular components such as membrane components, proteins, and lipids. Poly-nucleotides can be made by a cell and isolated using standard nucleic acid purification techniques, or synthesized using an amplification technique, such as the polymerase chain reaction (PCR), or by using an automatic synthesizer. Methods for isolating polynucleotides are routine and are known in the art. Any such technique

20   for obtaining a polynucleotide can be used to obtain isolated BMP-2 inducible kinase polynucleotides. For example, restriction enzymes and probes can be used to isolate polynucleotide fragments, which comprise BMP-2 inducible kinase nucleotide sequences. Isolated polynucleotides are in preparations that are free or at least 70, 80, or 90% free of other molecules.

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Human BMP-2 inducible kinase cDNA molecules can be made with standard molecular biology techniques, using BMP-2 inducible kinase mRNA as a template. Human BMP-2 inducible kinase cDNA molecules can thereafter be replicated using molecular biology techniques known in the art and disclosed in manuals such as

30   Sambrook *et al.* (1989). An amplification technique, such as PCR, can be used to

obtain additional copies of polynucleotides of the invention, using either human genomic DNA or cDNA as a template.

Alternatively, synthetic chemistry techniques can be used to synthesize BMP-2 inducible kinase polynucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a human BMP-2 inducible kinase polypeptide having, for example, an amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof.

Extending polynucleotides

Various PCR-based methods can be used to extend the nucleic acid sequences disclosed herein to detect upstream sequences such as promoters and regulatory elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus. Sarkar, *PCR Methods Applic. 2,* 318-322, 1993; Triglia *et al., Nucleic Acids Res. 16,* 8186, 1988; Lagerstrom *et al., PCR Methods Applic. 1,* 111-119, 1991; Parker *et al., Nucleic Acids Res. 19,* 3055-3060, 1991). Additionally, PCR, nested primers, and PROMOTERFINDER libraries (CLONTECH, Palo Alto, Calif.) can be used to walk genomic DNA (CLONTECH, Palo Alto, Calif.). See WO 01/98340

Obtaining Polynucleotides

Human BMP-2 inducible kinase polypeptides can be obtained, for example, by purification from human cells, by expression of BMP-2 inducible kinase poly-nucleotides, or by direct chemical synthesis.

Protein purification

Human BMP-2 inducible kinase polypeptides can be purified from any human cell which expresses the receptor, including host cells which have been transfected with

BMP-2 inducible kinase polynucleotides. A purified BMP-2 inducible kinase polypeptide is separated from other compounds that normally associate with the BMP-2 inducible kinase polypeptide in the cell, such as certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis.

A preparation of purified BMP-2 inducible kinase polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis.

Expression of polynucleotides

To express a human BMP-2 inducible kinase polynucleotide, the polynucleotide can be inserted into an expression vector that contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods that are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding BMP-2 inducible kinase polypeptides and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook *et al.* (1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

A variety of expression vector/host systems can be utilized to contain and express sequences encoding a human BMP-2 inducible kinase polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus

expression vectors (*e.g.*, baculovirus), plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems. See WO 01/98340.

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Host cells

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed BMP-2 inducible kinase polypeptide
10   in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells that have specific cellular machinery and characteristic
15   mechanisms for post-translational activities (*e.g.*, CHO, HeLa, MDCK, HEK293, and WI38) are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein. See WO 01/98340.

20   Alternatively, host cells which contain a human BMP-2 inducible kinase poly-nucleotide and which express a human BMP-2 inducible kinase polypeptide can be identified by a variety of procedures known to those of skill in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). Hampton *et al.*, SEROLOGICAL
25   METHODS: A LABORATORY MANUAL, APS Press, St. Paul, Minn., 1990) and Maddox *et al.*, *J. Exp. Med. 158*, 1211-1216, 1983). See WO 01/98340.

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for
30   producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding BMP-2 inducible kinase polypeptides include:

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oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding a human BMP-2 inducible kinase polypeptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Expression and purification of polypeptides

Host cells transformed with nucleotide sequences encoding a human BMP-2 inducible kinase polypeptide can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode BMP-2 inducible kinase polypeptides can be designed to contain signal sequences which direct secretion of soluble BMP-2 inducible kinase polypeptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound BMP-2 inducible kinase polypeptide. See WO 01/98340.

Chemical synthesis

Sequences encoding a human BMP-2 inducible kinase polypeptide can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*, *Nucl. Acids Res. Symp. Ser.* 215-223, 1980; Horn *et al. Nucl. Acids Res. Symp. Ser.* 225-232, 1980). Alternatively, a human BMP-2 inducible kinase

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polypeptide itself can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc. 85*, 2149-2154, 1963; Roberge *et al., Science 269*, 202-204, 1995). Protein synthesis can be performed using manual techniques or by

5   automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of BMP-2 inducible kinase polypeptides can be separately synthesized and combined using chemical methods to produce a full-length molecule. See WO 01/98340.

10   As will be understood by those of skill in the art, it may be advantageous to produce BMP-2 inducible kinase polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a

15   half-life, which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter BMP-2 inducible kinase polypeptide-encoding

20   sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new

25   restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

Antibodies

30   Any type of antibody known in the art can be generated to bind specifically to an epitope of a human BMP-2 inducible kinase polypeptide. "Antibody" as used herein

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includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab, F(ab')$_2$, and Fv, which are capable of binding an epitope of a human BMP-2 inducible kinase polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, e.g., at least 15, 25, or 50 amino acids.

An antibody which specifically binds to an epitope of a human BMP-2 inducible kinase polypeptide can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody that specifically binds to the immunogen.

Typically, an antibody that specifically binds to a human BMP-2 inducible kinase polypeptide provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, antibodies that specifically bind to BMP-2 inducible kinase polypeptides do not detect other proteins in immunochemical assays and can immunoprecipitate a human BMP-2 inducible kinase polypeptide from solution. See WO 01/98340.

Antisense oligonucleotides

Antisense oligonucleotides are nucleotide sequences that are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45,

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or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of BMP-2 inducible kinase gene products in the cell.

5

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such

10   alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. *See* Brown, *Meth. Mol. Biol. 20*, 1-8, 1994; Sonveaux, *Meth. Mol. Biol. 26*, 1-72, 1994; Uhlmann *et al.*, *Chem. Rev. 90*, 543-583, 1990.

15

Modifications of BMP-2 inducible kinase gene expression can be obtained by designing antisense oligonucleotides that will form duplexes to the control, 5', or regulatory regions of the BMP-2 inducible kinase gene. Oligonucleotides derived from the transcription initiation site, *e.g.*, between positions -10 and +10 from the

20   start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (*e.g.*, Gee *et al.*, in Huber & Carr, MOLECULAR AND

25   IMMUNOLOGIC APPROACHES, Futura Publishing Co., Mt. Kisco, N.Y., 1994). An antisense oligonucleotide also can be designed to block translation of mRNA by preventing the transcript from binding to ribosomes. See WO 01/98340.

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Ribozymes

Ribozymes are RNA molecules with catalytic activity. *See, e.g.*, Cech, *Science 236*, 1532-1539; 1987; Cech, *Ann. Rev. Biochem. 59*, 543-568; 1990, Cech, *Curr. Opin. Struct. Biol. 2*, 605-609; 1992, Couture & Stinchcomb, *Trends Genet. 12*, 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (*e.g.*, Haseloff *et al.*, U.S. Patent 5,641,673). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences.

The coding sequence of a human BMP-2 inducible kinase polynucleotide can be used to generate ribozymes that will specifically bind to mRNA transcribed from the BMP-2 inducible kinase polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (*see* Haseloff *et al.* *Nature 334*, 585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et al.*, EP 321,201). See WO 01/98340.

Differentially expressed genes

Described herein are methods for the identification of genes whose products interact with human BMP-2 inducible kinase. Such genes may represent genes that are differentially expressed in disorders including, but not limited to, cancer, diabetes, CNS disorders, COPD, gastrointestinal disorders, and cardiovascular disorders. Further, such genes may represent genes that are differentially regulated in response

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to manipulations relevant to the progression or treatment of such diseases. Additionally, such genes may have a temporally modulated expression, increased or decreased at different stages of tissue or organism development. A differentially expressed gene may also have its expression modulated under control versus experimental conditions. In addition, the human BMP-2 inducible kinase gene or gene product may itself be tested for differential expression.

The degree to which expression differs in a normal versus a diseased state need only be large enough to be visualized via standard characterization techniques such as differential display techniques. Other such standard characterization techniques by which expression differences may be visualized include but are not limited to, quantitative RT (reverse transcriptase), PCR, and Northern analysis.

To identify differentially expressed genes total RNA or, preferably, mRNA is isolated from tissues of interest. For example, RNA samples are obtained from tissues of experimental subjects and from corresponding tissues of control subjects. Any RNA isolation technique that does not select against the isolation of mRNA may be utilized for the purification of such RNA samples. See, for example, Ausubel *et al.*, ed., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, Inc. New York, 1987-1993. Large numbers of tissue samples may readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski, U.S. Patent 4,843,155.

Transcripts within the collected RNA samples that represent RNA produced by differentially expressed genes are identified by methods well known to those of skill in the art. They include, for example, differential screening (Tedder *et al., Proc. Natl. Acad. Sci. U.S.A. 85*, 208-12, 1988), subtractive hybridization (Hedrick *et al., Nature 308*, 149-53; Lee *et al., Proc. Natl. Acad. Sci. U.S.A. 88*, 2825, 1984), and, preferably, differential display (Liang & Pardee, *Science 257*, 967-71, 1992; U.S. Patent 5,262,311).

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The differential expression information may itself suggest relevant methods for the treatment of disorders involving the human BMP-2 inducible kinase. For example, treatment may include a modulation of expression of the differentially expressed genes and/or the gene encoding the human BMP-2 inducible kinase. The differential expression information may indicate whether the expression or activity of the differentially expressed gene or gene product or the human BMP-2 inducible kinase gene or gene product are up-regulated or down-regulated.

Screening methods

The invention provides assays for screening test compounds that bind to or modulate the activity of a human BMP-2 inducible kinase polypeptide or a human BMP-2 inducible kinase polynucleotide. A test compound preferably binds to a human BMP-2 inducible kinase polypeptide or polynucleotide. More preferably, a test compound decreases or increases enzymatic activity by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

Test compounds

Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches

are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. *See* Lam, *Anticancer Drug Des. 12*, 145, 1997.

Methods for the synthesis of molecular libraries are well known in the art (*see*, for example, DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 90*, 6909, 1993; Erb *et al. Proc. Natl. Acad. Sci. U.S.A. 91*, 11422, 1994; Zuckermann *et al.*, *J. Med. Chem. 37*, 2678, 1994; Cho *et al.*, *Science 261*, 1303, 1993; Carell *et al.*, *Angew. Chem. Int. Ed. Engl. 33*, 2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl. 33*, 2061; Gallop *et al.*, *J. Med. Chem. 37*, 1233, 1994). Libraries of compounds can be presented in solution (*see, e.g.*, Houghten, *BioTechniques 13*, 412-421, 1992), or on beads (Lam, *Nature 354*, 82-84, 1991), chips (Fodor, *Nature 364*, 555-556, 1993), bacteria or spores (Ladner, U.S. Patent 5,223,409), plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 89*, 1865-1869, 1992), or phage (Scott & Smith, *Science 249*, 386-390, 1990; Devlin, *Science 249*, 404-406, 1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci. 97*, 6378-6382, 1990; Felici, *J. Mol. Biol. 222*, -310, 1991; and Ladner, U.S. Patent 5,223,409).

High throughput screening

Test compounds can be screened for the ability to bind to BMP-2 inducible kinase polypeptides or polynucleotides or to affect BMP-2 inducible kinase activity or BMP-2 inducible kinase gene expression using high throughput screening. Using high throughput screening, many discrete compounds can be tested in parallel so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 50 to 500 μl. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the 96-well format.

Alternatively, "free format assays," or assays that have no physical barrier between samples, can be used. For example, an assay using pigment cells (melanocytes) in a simple homogeneous assay for combinatorial peptide libraries is described by

Jayawickreme *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 19*, 1614-18 (1994). The cells are placed under agarose in petri dishes, then beads that carry combinatorial compounds are placed on the surface of the agarose. The combinatorial compounds are partially released the compounds from the beads. Active compounds can be visualized as

5   dark pigment areas because, as the compounds diffuse locally into the gel matrix, the active compounds cause the cells to change colors.

Another example of a free format assay is described by Chelsky, "Strategies for Screening Combinatorial Libraries: Novel and Traditional Approaches," reported at

10   the First Annual Conference of The Society for Biomolecular Screening in Philadelphia, Pa. (Nov. 7-10, 1995). Chelsky placed a simple homogenous enzyme assay for carbonic anhydrase inside an agarose gel such that the enzyme in the gel would cause a color change throughout the gel. Thereafter, beads carrying combinatorial compounds via a photolinker were placed inside the gel and the

15   compounds were partially released by UV-light. Compounds that inhibited the enzyme were observed as local zones of inhibition having less color change.

Yet another example is described by Salmon *et al.*, *Molecular Diversity 2*, 57-63 (1996). In this example, combinatorial libraries were screened for compounds that

20   had cytotoxic effects on cancer cells growing in agar.

Another high throughput screening method is described in Beutel *et al.*, U.S. Patent 5,976,813. In this method, test samples are placed in a porous matrix. One or more assay components are then placed within, on top of, or at the bottom of a matrix such

25   as a gel, a plastic sheet, a filter, or other form of easily manipulated solid support. When samples are introduced to the porous matrix they diffuse sufficiently slowly, such that the assays can be performed without the test samples running together.

Binding assays

For binding assays, the test compound is preferably a small molecule that binds to and occupies, for example, the active site of the BMP-2 inducible kinase polypeptide,

5   such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules.

In binding assays, either the test compound or the BMP-2 inducible kinase polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic,

10   chemiluminescent, or enzymatic label, such as horseradish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound that is bound to the BMP-2 inducible kinase polypeptide can then be accomplished, for example, by direct counting of radioemmission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

15

Alternatively, binding of a test compound to a human BMP-2 inducible kinase polypeptide can be determined without labeling either of the interactants. For example, a microphysiometer can be used to detect binding of a test compound with a human BMP-2 inducible kinase polypeptide. A microphysiometer (*e.g.*,

20   Cytosensor™) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and a human BMP-2 inducible kinase polypeptide (McConnell *et al.*, *Science 257*, 1906-1912, 1992).

25

Determining the ability of a test compound to bind to a human BMP-2 inducible kinase polypeptide also can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, *Anal. Chem. 63*, 2338-2345, 1991, and Szabo *et al.*, *Curr. Opin. Struct. Biol. 5*, 699-705, 1995). BIA

30   is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore™). Changes in the optical phenomenon surface

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plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another aspect of the invention, a human BMP-2 inducible kinase polypeptide can be used as a "bait protein" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Patent 5,283,317; Zervos et al., Cell 72, 223-232, 1993; Madura et al., J. Biol. Chem. 268, 12046-12054, 1993; Bartel et al., BioTechniques 14, 920-924, 1993; Iwabuchi et al., Oncogene 8, 1693-1696, 1993; and Brent WO94/10300), to identify other proteins which bind to or interact with the BMP-2 inducible kinase polypeptide and modulate its activity.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. For example, in one construct, polynucleotide encoding a human BMP-2 inducible kinase polypept... ... be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct a DNA sequence that encodes an unidentified protein ("prey" or "sample") can be fused to a polynucleotide that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact in vivo to form an protein-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ), which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor can be isolated and used to obtain the DNA sequence encoding the protein that interacts with the BMP-2 inducible kinase polypeptide.

It may be desirable to immobilize either the BMP-2 inducible kinase polypeptide (or polynucleotide) or the test compound to facilitate separation of bound from unbound forms of one or both of the interactants, as well as to accommodate automation of the

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assay. Thus, either the BMP-2 inducible kinase polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex,

5   polystyrene, or glass beads). Any method known in the art can be used to attach the polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-covalent linkages, passive absorption, or pairs of binding moieties attached respectively to the polypeptide (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array,

10 so that the location of individual test compounds can be tracked. Binding of a test compound to a human BMP-2 inducible kinase polypeptide (or polynucleotide) can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

15 In one embodiment, the BMP-2 inducible kinase polypeptide is a fusion protein comprising a domain that allows the BMP-2 inducible kinase polypeptide to be bound to a solid support. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test

20 compound or the test compound and the non-adsorbed BMP-2 inducible kinase polypeptide; the mixture is then incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as

25 described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a human

30 BMP-2 inducible kinase polypeptide (or polynucleotide) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated BMP-2

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inducible kinase polypeptides (or polynucleotides) or test compounds can be prepared from biotin-NHS(N- hydroxysuccinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which specifically bind to a BMP-2 inducible kinase polypeptide, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of the BMP-2 inducible kinase polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the BMP-2 inducible kinase polypeptide or test compound, enzyme-linked assays which rely on detecting an activity of the BMP-2 inducible kinase polypeptide, and SDS gel electrophoresis under non-reducing conditions.

Screening for test compounds which bind to a human BMP-2 inducible kinase polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a BMP-2 inducible kinase polypeptide or polynucleotide can be used in a cell-based assay system. A BMP-2 inducible kinase polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to a BMP-2 inducible kinase polypeptide or polynucleotide is determined as described above.

Enzymatic activity

Test compounds can be tested for the ability to increase or decrease the enzymatic activity of a human BMP-2 inducible kinase polypeptide. Enzymatic activity can be measured, for example, as described in Example 4.

Enzyme assays can be carried out after contacting either a purified BMP-2 inducible kinase polypeptide, a cell membrane preparation, or an intact cell with a test compound. A test compound that decreases enzymatic activity of a human BMP-2 inducible kinase polypeptide by at least about 10, preferably about 50, more
5   preferably about 75, 90, or 100% is identified as a potential therapeutic agent for decreasing BMP-2 inducible kinase activity. A test compound that increases enzymatic activity of a human BMP-2 inducible kinase polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for increasing human BMP-2 inducible kinase activity.

10

Gene expression

In another embodiment, test compounds that increase or decrease BMP-2 inducible kinase gene expression are identified. A BMP-2 inducible kinase polynucleotide is
15   contacted with a test compound, and the expression of an RNA or polypeptide product of the BMP-2 inducible kinase polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of
20   expression based on this comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide is less in the presence of the test compound than in its absence, the test compound is identified
25   as an inhibitor of the mRNA or polypeptide expression.

The level of BMP-2 inducible kinase mRNA or polypeptide expression in the cells can be determined by methods well known in the art for detecting mRNA or polypeptide. Either qualitative or quantitative methods can be used. The presence of
30   polypeptide products of a human BMP-2 inducible kinase polynucleotide can be determined, for example, using a variety of techniques known in the art, including

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immunochemical methods such as radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined *in vivo*, in a cell culture, or in an *in vitro* translation system by detecting incorporation of labeled amino acids into a human BMP-2 inducible kinase polypeptide.

5

Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell that expresses a human BMP-2 inducible kinase polynucleotide can be used in a cell-based assay system. The BMP-2 inducible kinase polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those

10   described above. Either a primary culture or an established cell line, such as CHO or human embryonic kidney 293 cells, can be used.

Pharmaceutical compositions

15   The invention also provides pharmaceutical compositions that can be administered to a patient to achieve a therapeutic effect. Pharmaceutical compositions of the invention can comprise, for example, a human BMP-2 inducible kinase polypeptide, BMP-2 inducible kinase polynucleotide, ribozymes or antisense oligonucleotides, antibodies which specifically bind to a BMP-2 inducible kinase polypeptide, or

20   mimetics, activators, or inhibitors of a human BMP-2 inducible kinase polypeptide activity. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a

25   patient alone, or in combination with other agents, drugs or hormones.

In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be

30   used pharmaceutically. Pharmaceutical compositions of the invention can be administered by any number of routes including, but not limited to, oral, intravenous,

intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores can be used in conjunction with suitable coatings, such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds can be

dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions can contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers also can be used for delivery. Optionally, the suspension also can contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. For topical or local administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention can be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The pharmaceutical composition can be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation can be a lyophilized powder which can contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co.,

Easton, Pa.). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

5

Therapeutic indications and methods

Human BMP-2 inducible kinase can be regulated to treat cancer, diabetes, CNS disorders, COPD, gastrointestinal disorders, and cardiovascular disorders.

10

Cancer

The novel human BMP-2 inducible kinase is highly expressed in the following cancer tissues: esophageal tumor, and tumors of the ileum, colon, and stomach. The
15   expression in the above mentioned tissues and in particular the differential expression between diseased tissue (esophageal tumor) and healthy tissue (esophagus), between diseased tissue (tumors of the ileum, colon, and stomach) and healthy tissue (ileum, colon, and stomach) demonstrates that the novel human BMP-2 inducible kinase or mRNA can be used to diagnose cancer. Additionally, the activity
20   of the novel human BMP-2 inducible kinase can be modulated to treat cancer.

Cancer disorders within the scope of the invention comprise any disease of an organ or tissue in mammals characterized by poorly controlled or uncontrolled multiplication of normal or abnormal cells in that tissue and its effect on the body as
25   a whole. Cancer diseases within the scope of the invention comprise benign neoplasms, dysplasias, hyperplasias as well as neoplasms showing metastatic growth or any other transformations, *e.g.*, leukoplakias, which often precede a breakout of cancer. Cells and tissues are cancerous when they grow more rapidly than normal cells, displacing or spreading into the surrounding healthy tissue or any other tissues
30   of the body described as metastatic growth, assume abnormal shapes and sizes, show

changes in their nucleocytoplasmatic ratio, nuclear polychromasia, and finally may
cease.

Cancerous cells and tissues may affect the body as a whole when causing
paraneoplastic syndromes or if cancer occurs within a vital organ or tissue, normal
function will be impaired or halted, with possible fatal results. The ultimate
involvement of a vital organ by cancer, either primary or metastatic, may lead to the
death of the mammal affected. Cancer tends to spread, and the extent of its spread is
usually related to an individual's chances of surviving the disease. Cancers are
generally said to be in one of three stages of growth: early, or localized, when a
tumor is still confined to the tissue of origin, or primary site; direct extension, where
cancer cells from the tumor have invaded adjacent tissue or have spread only to
regional lymph nodes; or metastasis, in which cancer cells have migrated to distant
parts of the body from the primary site, via the blood or lymph systems, and have
established secondary sites of infection. Cancer is said to be malignar⁻ ⁻ ⁻se of its
tendency to cause death if not treated.

Benign tumors usually do not cause death, although they may if they interfere with a
normal body function by virtue of their location, size, or paraneoplastic side effects.
Hence, benign tumors fall under the definition of cancer within the scope of the
invention as well. In general, cancer cells divide at a higher rate than do normal cells,
but the distinction between the growth of cancerous and normal tissues is not so
much the rapidity of cell division in the former as it is the partial or complete loss of
growth restraint in cancer cells and their failure to differentiate into a useful, limited
tissue of the type that characterizes the functional equilibrium of growth of normal
tissue.

Cancer tissues may express certain molecular receptors and probably are influenced
by the host's susceptibility and immunity and it is known that certain cancers of the
breast and prostate, for example, are considered dependent on specific hormones for
their existence. The term "cancer" under the scope of the invention is not limited to

simple benign neoplasia but includes any other benign and malign neoplasia, such as 1) carcinoma, 2) sarcoma, 3) carcinosarcoma, 4) cancers of the blood-forming tissues, 5) tumors of nerve tissues including the brain, and 6) cancer of skin cells.

Carcinoma occurs in epithelial tissues, which cover the outer body (the skin) and line mucous membranes and the inner cavitary structures of organs e.g. such as the breast, lung, the respiratory and gastrointestinal tracts, the endocrine glands, and the genitourinary system. Ductal or glandular elements may persist in epithelial tumors, as in adenocarcinomas, e.g., thyroid adenocarcinoma, gastric adenocarcinoma, and uterine adenocarcinoma. Cancers of the pavement-cell epithelium of the skin and of certain mucous membranes, such as cancers of the tongue, lip, larynx, urinary bladder, uterine cervix, or penis, may be termed epidermoid or squamous-cell carcinomas of the respective tissues and are within the scope of the definition of cancer as well.

Sarcomas develop in connective tissues, including fibrous tissues, adipose (fat) tissues, muscle, blood vessels, bone, and cartilage such as osteogenic sarcoma, liposarcoma, fibrosarcoma, and synovial sarcoma.

Carcinosarcoma is cancer that develops in both epithelial and connective tissue. Cancer disease within the scope of this definition may be primary or secondary, whereby primary indicates that the cancer originated in the tissue where it is found rather than was established as a secondary site through metastasis from another lesion. Cancers and tumor diseases within the scope of this definition may be benign or malign and may affect all anatomical structures of the body of a mammal. By example, to they comprise cancers and tumor diseases of I) the bone marrow and bone marrow derived cells (leukemias), II) the endocrine and exocrine glands, such as the thyroid, parathyroid, pituitary, adrenal glands, salivary glands, and pancreas III) the breast, such as benign or malignant tumors in the mammary glands of either a male or a female, the mammary ducts, adenocarcinoma, medullary carcinoma, comedocarcinoma, Paget's disease of the nipple, inflammatory carcinoma of the

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young woman, IV) the lung, V) the stomach, VI) the liver and spleen, VII) the small intestine, VIII) the colon, IX) the bone and its supportive and connective tissues such as malignant or benign bone tumor, such as malignant osteogenic sarcoma, benign osteoma, cartilage tumors, malignant chondrosarcoma or benign chondroma; bone marrow tumors such as malignant myeloma or benign eosinophilic granuloma, as well as metastatic tumors from bone tissues at other locations of the body; X) the mouth, throat, larynx, and the esophagus, XI) the urinary bladder and the internal and external organs and structures of the urogenital system of male and female such as the ovaries, uterus, cervix of the uterus, testes, and prostate gland, XII) the prostate, XIII) the pancreas, such as ductal carcinoma of the pancreas; XIV) the lymphatic tissue such as lymphomas and other tumors of lymphoid origin, XV) the skin, XVI) cancers and tumor diseases of all anatomical structures belonging to the respiratory systems including thoracal muscles and linings, XVII) primary or secondary cancer of the lymph nodes, XVIII) the tongue and of the bony structures of the hard palate or sinuses, XVIV) the mouth, cheeks, neck and salivary glands, XX) the blood vessels including the heart and their linings, XXI) the smooth or skeletal muscles and their ligaments and linings, XXII) the peripheral, the autonomous, the central nervous system including the cerebellum, and XXIII) the adipose tissue.

Cancer is a disease fundamentally caused by oncogenic cellular transformation. There are several hallmarks of transformed cells that distinguish them from their normal counterparts and underlie the pathophysiology of cancer. These include uncontrolled cellular proliferation, unresponsiveness to normal death-inducing signals (immortalization), increased cellular motility and invasiveness, increased ability to recruit blood supply through induction of new blood vessel formation (angiogenesis), genetic instability, and dysregulated gene expression. Various combinations of these aberrant physiologies, along with the acquisition of drug-resistance frequently lead to an intractable disease state in which organ failure and patient death ultimately ensue.

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Most standard cancer therapies target cellular proliferation and rely on the differential proliferative capacities between transformed and normal cells for their efficacy. This approach is hindered by the facts that several important normal cell types are also highly proliferative and that cancer cells frequently become resistant to these agents.

5   Thus, the therapeutic indices for traditional anti-cancer therapies rarely exceed 2.0.

The advent of genomics-driven molecular target identification has opened up the possibility of identifying new cancer-specific targets for therapeutic intervention that will provide safer, more effective treatments for cancer patients. Thus, newly

10   discovered tumor-associated genes and their products can be tested for their role(s) in disease and used as tools to discover and develop innovative therapies. Genes playing important roles in any of the physiological processes outlined above can be characterized as cancer targets.

Genes or gene fragments identified through genomics can readily be expressed in one or more heterologous expression systems to produce functional recombinant proteins. These proteins are characterized *in vitro* for their biochemical properties and then used as tools in high-throughput molecular screening programs to identify chemical modulators of their biochemical activities. Agonists and/or antagonists of target

20   protein activity can be identified in this manner and subsequently tested in cellular and *in vivo* disease models for anti-cancer activity. Optimization of lead compounds with iterative testing in biological models and detailed pharmacokinetic and toxicological analyses form the basis for drug development and subsequent testing in humans.

25.

Protein kinases and cancer treatment

Protein phosphorylation is an essential component in intracellular signaling, with diverse and crucial functions including mediation of cell proliferation, survival,

.30   apoptosis, differentiation, migration and attachment. It is regulated by the balance between the opposing activities of protein kinases and protein phosphatases. Protein

phosphorylation is mainly mediated by two types of protein kinases – protein tyrosine kinases and protein serine/threonine kinases. A number of protein tyrosine kinases are encoded by proto-oncogenes or viral oncogenes, and are thus strongly implicated in cancer. Protein serine/threonine kinases are known to play a role in intracellular
5   signal transduction mediated by growth factors, cytokines, etc. inducing either cell proliferation, apoptosis or differentiation. Inhibitors of protein kinases are expected to provide efficacious therapeutic agents for the treatment of cancer.

Diabetes

10

Diabetes mellitus is a common metabolic disorder characterized by an abnormal elevation in blood glucose, alterations in lipids and abnormalities (complications) in the cardiovascular system, eye, kidney and nervous system. Diabetes is divided into two separate diseases: type 1 diabetes (juvenile onset), which results from a loss of
15   cells which make and secrete insulin, and type 2 diabetes (adult onset) which is caused by a defect in insulin secretion and a defect in insulin action.

Type I diabetes is initiated by an autoimmune reaction that attacks the insulin secreting cells (beta cells) in the pancreatic islets. Agents that prevent this reaction
20   from occurring or that stop the reaction before destruction of the beta cells has been accomplished are potential therapies for this disease. Other agents that induce beta cell proliferation and regeneration also are potential therapies.

Type II diabetes is the most common of the two diabetic conditions (6% of the
25   population). The defect in insulin secretion is an important cause of the diabetic condition and results from an inability of the beta cell to properly detect and respond to rises in blood glucose levels with insulin release. Therapies that increase the response by the beta cell to glucose would offer an important new treatment for this disease.

30

The defect in insulin action in Type II diabetic subjects is another target for therapeutic intervention. Agents that increase the activity of the insulin receptor in muscle, liver, and fat will cause a decrease in blood glucose and a normalization of plasma lipids. The receptor activity can be increased by agents that directly stimulate

5   the receptor or that increase the intracellular signals from the receptor. Other therapies can directly activate the cellular end process, *i.e.* glucose transport or various enzyme systems, to generate an insulin-like effect and therefore a produce beneficial outcome. Because overweight subjects have a greater susceptibility to Type II diabetes, any agent that reduces body weight is a possible therapy.

10

Both Type I and Type II diabetes can be treated with agents that mimic insulin action or that treat diabetic complications by reducing blood glucose levels. Likewise, agents that reduces new blood vessel growth can be used to treat the eye complications that develop in both diseases.

15

CNS disorders

The novel human BMP-2 inducible kinase is highly expressed in the following brain tissues: occipital lobe, parietal lobe, temporal lobe, hippocampus, Alzheimer cerebral cortex, spinal cord, thalamus, cerebral peduncles, substantia nigra, vermis cerebelli,

20   precentral gyrus, Alzheimer brain frontal lobe, Alzheimer brain, cerebral cortex, cerebellum (left), corpus callosum, cerebellum (right), brain, fetal brain, frontal lobe, retina. The expression in brain tissues and in particular the differential expression between diseased tissue Alzheimer cerebral cortex and healthy tissue cerebral cortex, between diseased tissue Alzheimer brain frontal lobe and healthy tissue frontal lobe,

25   between diseased tissue Alzheimer brain and healthy tissue brain demonstrates that the novel human BMP-2 inducible kinase or mRNA can be used to diagnose nervous system diseases. Additionally, the activity of the novel human BMP-2 inducible kinase can be modulated to treat nervous system diseases.

30

Central and peripheral nervous system disorders also can be treated, such as primary and secondary disorders after brain injury, disorders of mood, anxiety disorders, disorders of thought and volition, disorders of sleep and wakefulness, diseases of the motor unit, such as neurogenic and myopathic disorders, neurodegenerative disorders such as Alzheimer's and Parkinson's disease, and processes of peripheral and chronic pain.

Pain that is associated with CNS disorders also can be treated by regulating the activity of human BMP-2 inducible kinase. Pain which can be treated includes that associated with central nervous system disorders, such as multiple sclerosis, spinal cord injury, sciatica, failed back surgery syndrome, traumatic brain injury, epilepsy, Parkinson's disease, post-stroke, and vascular lesions in the brain and spinal cord (e.g., infarct, hemorrhage, vascular malformation). Non-central neuropathic pain includes that associated with post mastectomy pain, reflex sympathetic dystrophy (RSD), trigeminal algiaradioculopathy, post-surgical pain, HIV/AIDS related pain, cancer pain, metabolic neuropathies (e.g., diabetic neuropathy, vasculitic neuropathy secondary to connective tissue disease), paraneoplastic polyneuropathy associated, for example, with carcinoma of lung, or leukemia, or lymphoma, or carcinoma of prostate, colon or stomach, trigeminal neuralgia, cranial neuralgias, and post-herpetic neuralgia. Pain associated with cancer and cancer treatment also can be treated, as can headache pain (for example, migraine with aura, migraine without aura, and other migraine disorders), episodic and chronic tension-type headache, tension-type like headache, cluster headache, and chronic paroxysmal hemicrania.

COPD

Chronic obstructive pulmonary (or airways) disease (COPD) is a condition defined physiologically as airflow obstruction that generally results from a mixture of emphysema and peripheral airway obstruction due to chronic bronchitis (Senior & Shapiro, *Pulmonary Diseases and Disorders*, 3d ed., New York, McGraw-Hill, 1998, pp. 659-681, 1998; Barnes, *Chest 117*, 10S-14S, 2000). Emphysema is characterized

by destruction of alveolar walls leading to abnormal enlargement of the air spaces of the lung. Chronic bronchitis is defined clinically as the presence of chronic productive cough for three months in each of two successive years. In COPD, airflow obstruction is usually progressive and is only partially reversible. By far the most important risk factor for development of COPD is cigarette smoking, although the disease does occur in non-smokers.

Chronic inflammation of the airways is a key pathological feature of COPD (Senior & Shapiro, 1998). The inflammatory cell population comprises increased numbers of macrophages, neutrophils, and CD8^{+} lymphocytes. Inhaled irritants, such as cigarette smoke, activate macrophages that are resident in the respiratory tract, as well as epithelial cells leading to release of chemokines (e.g., interleukin-8) and other chemotactic factors. These chemotactic factors act to increase the neutrophil/-monocyte trafficking from the blood into the lung tissue and airways. Neutrophils and monocytes recruited into the airways can release a variety of potentially damaging mediators such as proteolytic enzymes and reactive oxygen species. Matrix degradation and emphysema, along with airway wall thickening, surfactant dysfunction, and mucus hypersecretion, all are potential sequelae of this inflammatory response that lead to impaired airflow and gas exchange.

Protein kinases and treatment of COPD

Protein kinases are signal transducing enzymes that phosphorylate proteins, including other kinases, and, along with protein phosphatases, regulate the level and extent of protein phosphorylation and activation. Intracellular signalling pathways have important roles in inflammatory processes. These pathways may be activated by cytokines, oxidant stress and other inflammatory mediators (reviewed in Kyraikis & Avruch, *J. Biol. Chem.* *271*, 24313-16, 1996; Kyraikis & Avruch, *J. Physiol. Rev.* *81*, 807-69, 2001). For example, the pro-inflammatory cytokines, tumor necrosis factor α (TNFα) and interleukin-1 activate the protein ser/thr kinases c-Jun-NH2-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase, leading to

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activation of AP-1 and IKB kinase (IKK), which, in turn, leads to activation of the transcription factor NFKB. Activation of NFKB is required for the transcription of several pro-inflammatory molecules, including interleukin-8 and ICAM-1. Enzymes of the MAP kinase class may also act to increase cytokine production by stabilization of mRNA (Winzen *et al.*, *EMBO J. 18*, 4969980, 1999).

Inhibition of specific protein kinases has been shown to elicit anti-inflammatory effects. For example, the accumulation of polymorphonuclear leukocytes in murine lung following intratracheal administration of bacterial lipopolysaccharide can be blocked by inhibition of p38 MAP kinase (Nick *et al.*, *J. Immunol. 164*, 2151-59, 2000). As a further example, aerosol delivery to rat lungs of antisense oligodeoxynucleotides to syk kinase mRNA, suppressed nitric oxide and TNFα production from alveolar macrophages stimulated with IgG-anti-IgG complexes (Stenton *et al.*, *J. Immunol. 164*, 3790-97, 2000). Protein kinase subtypes are therefore attractive therapeutic targets for the attenuation of the inflammatory response in COPD.

Gastrointestinal disorders

The novel human BMP-2 inducible kinase is highly expressed in the following tissues of the gastrointestinal system: rectum, ileum, stomach, and esophagus, esophageal tumor, colon, tumors of the ileum, colon, and stomach,. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue (tumor of the ileum) and healthy tissue (ileum) demonstrates that the novel human BMP-2 inducible kinase or mRNA can be used to diagnose gastrointestinal disorders. Additionally, the activity of the novel human BMP-2 inducible kinase can be modulated to treat gastrointestinal disorders.

Gastrointestinal disorders comprise primary or secondary, acute or chronic diseases of the organs of the gastrointestinal tract which may be acquired or inherited, benign or malignant or metaplastic, and which may affect the organs of the gastrointestinal

tract or the body as a whole. They include but are not limited to disorders of the esophagus, such as achalasia, vigoruos achalasia, dysphagia, cricopharyngeal incoordination, pre-esophageal dysphagia, diffuse esophageal spasm, globus sensation, Barrett's metaplasia, and gastroesophageal reflux. They also include disorders of the stomach and duodenum, such as functional dyspepsia, inflammation of the gastric mucosa, gastritis, stress gastritis, chronic erosive gastritis, atrophy of gastric glands, metaplasia of gastric tissues, gastric ulcers, duodenal ulcers, and neoplasms of the stomach. Gastrointestinal disorders also include disorders of the pancreas, such as acute or chronic pancreatitis, insufficiency of the exocrinic or endocrinic tissues of the pancreas like steatorrhea, diabetes, neoplasms of the exocrine or endocrine pancreas (e.g., multiple endocrine neoplasia syndrome, ductal adenocarcinoma, cystadenocarcinoma, islet cell tumors, insulinoma, gastrinoma, carcinoid tumors, and glucagonoma), Zollinger-Ellison syndrome, Vipoma syndrome, and malabsorption syndrome. Gastrointestinal disorders also include disorders of the bowel, such as chronic inflammato ... diseases of the bowel, Crohn's disease, ileus, diarrhea and constipation, colonic ... ... , megacolon, malabsorption syndrome, and ulcerative colitis, functional bowel disorders, such as irritable bowel syndrome, neoplasms of the bowel, such as familial polyposis, adenocarcinoma, primary malignant lymphoma, carcinoid tumors, Kaposi's sarcoma, polyps, and cancer of the colon and rectum.

Cardiovascular disorders

The novel human BMP-2 inducible kinase is highly expressed in the following cardiovascular related tissues: heart atrium (right), fetal heart, HUVEC cells, heart atrium (left), interventricular septum, heart, and pericardium. Expression in the above-mentioned tissues demonstrates that the novel human BMP-2 inducible kinase or mRNA can be used to diagnose cardiovascular diseases. Additionally, the activity of the novel human BMP-2 inducible kinase can be modulated to treat cardiovascular diseases.

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Cardiovascular diseases include the following disorders of the heart and the vascular system: congestive heart failure, myocardial infarction, ischemic diseases of the heart, all kinds of atrial and ventricular arrhythmias, hypertensive vascular diseases, and peripheral vascular diseases.

5

Heart failure is defined as a pathophysiologic state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirement of the metabolizing tissue. It includes all forms of pumping failure, such as high-output and low-output, acute and chronic, right-

10   sided or left-sided, systolic or diastolic, independent of the underlying cause.

Myocardial infarction (MI) is generally caused by an abrupt decrease in coronary blood flow that follows a thrombotic occlusion of a coronary artery previously narrowed by arteriosclerosis. MI prophylaxis (primary and secondary prevention) is

15   included, as well as the treatment of MI and the prevention of complications.

Ischemic diseases are conditions in which the coronary flow is restricted resulting in a perfusion which inadequate to meet the myocardial requirement for oxygen. This group of diseases includes stable angina, unstable angina, and asymptomatic

20   ischemia.

Arrhythmias include all forms of atrial and ventricular tachyarrhythmias (atrial tachycardia, atrial flutter, atrial fibrillation, atrio-ventricular reentrant tachycardia, preexcitation syndrome, ventricular tachycardia, ventricular flutter, and ventricular

25   fibrillation), as well as bradycardic forms of arrhythmias.

Vascular diseases include primary as well as all kinds of secondary arterial hypertension (renal, endocrine, neurogenic, others). The disclosed gene and its product may be used as drug targets for the treatment of hypertension as well as for

30   the prevention of all complications. Peripheral vascular diseases are defined as vascular diseases in which arterial and/or venous flow is reduced resulting in an

imbalance between blood supply and tissue oxygen demand. It includes chronic peripheral arterial occlusive disease (PAOD), acute arterial thrombosis and embolism, inflammatory vascular disorders, Raynaud's phenomenon, and venous disorders.

5

This invention further pertains to the use of novel agents identified by the screening assays described above. Accordingly, it is within the scope of this invention to use a test compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein (e.g., a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a human BMP-2 inducible kinase polypeptide binding molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

A reagent which affects BMP-2 inducible kinase activity can be administered to a human cell, either in vitro or in vivo, to reduce BMP-2 inducible kinase activity. The reagent preferably binds to an expression product of a human BMP-2 inducible kinase gene. If the expression product is a protein, the reagent is preferably an antibody. For treatment of human cells ex vivo, an antibody can be added to a preparation of stem cells that have been removed from the body. The cells can then be replaced in the same or another human body, with or without clonal propagation, as is known in the art.

In one embodiment, the reagent is delivered using a liposome. Preferably, the liposome is stable in the animal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of targeting a reagent, particularly a polynucleotide, to a particular site in an animal,

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such as a human. Preferably, the lipid composition of the liposome is capable of targeting to a specific organ of an animal, such as the lung, liver, spleen, heart brain, lymph nodes, and skin.

5   A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its contents to the cell. Preferably, the transfection efficiency of a liposome is about 0.5 μg of DNA per 16 nmole of liposome delivered to about 10^6 cells, more preferably about 1.0 μg of DNA per 16 nmole of liposome delivered to about 10^6

10   cells, and even more preferably about 2.0 μg of DNA per 16 nmol of liposome delivered to about 10^6 cells. Preferably, a liposome is between about 100 and 500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

15   Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Optionally, a liposome comprises a compound capable of

20   targeting the liposome to a particular cell type, such as a cell-specific ligand exposed on the outer surface of the liposome.

Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods that are standard in the art (see, for

25   example, U.S. Patent 5,705,151). Preferably, from about 0.1 μg to about 10 μg of polynucleotide is combined with about 8 nmol of liposomes, more preferably from about 0.5 μg to about 5 μg of polynucleotides are combined with about 8 nmol liposomes, and even more preferably about 1.0 μg of polynucleotides is combined with about 8 nmol liposomes.

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In another embodiment, antibodies can be delivered to specific tissues *in vivo* using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques are taught in, for example, Findeis *et al. Trends in Biotechnol. 11*, 202-05 (1993); Chiou *et al.*, GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE
5   TRANSFER (J.A. Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem. 263*, 621-24 (1988); Wu *et al., J. Biol. Chem. 269*, 542-46 (1994); Zenke *et al., Proc. Natl. Acad. Sci. U.S.A. 87*, 3655-59 (1990); Wu *et al., J. Biol. Chem. 266*, 338-42 (1991).

Determination of a therapeutically effective dose

10
The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient which increases or decreases enzymatic activity relative to the enzymatic activity which occurs in the absence of the therapeutically effective dose.

15
For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses
20   and routes for administration in humans.

Therapeutic efficacy and toxicity, *e.g.*, ED_{50} (the dose therapeutically effective in 50% of the population) and LD_{50} (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental
25   animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD_{50}/ED_{50}.

Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a
30   range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED_{50} with

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little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors that can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upc...'': route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

If the reagent is a single-chain antibody, polynucleotides encoding the antibody can be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium phosphate-mediated transfection.

Effective *in vivo* dosages of an antibody are in the range of about 5 μg to about 50 μg/kg, about 50 μg to about 5 mg/kg, about 100 μg to about 500 μg/kg of patient

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body weight, and about 200 to about 250 µg/kg of patient body weight. For administration of polynucleotides encoding single-chain antibodies, effective *in vivo* dosages are in the range of about 100 ng to about 200 ng, 500 ng to about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about

5   100 µg of DNA.

If the expression product is mRNA, the reagent is preferably an antisense oligo-nucleotide or a ribozyme. Polynucleotides that express antisense oligonucleotides or ribozymes can be introduced into cells by a variety of methods, as described above.

10

Preferably, a reagent reduces expression of a human BMP-2 inducible kinase gene or the activity of a BMP-2 inducible kinase polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the reagent. The effectiveness of the mechanism chosen to decrease the level of

15   expression of a human BMP-2 inducible kinase gene or the activity of a human BMP-2 inducible kinase polypeptide can be assessed using methods well known in the art, such as hybridization of nucleotide probes to BMP-2 inducible kinase-specific mRNA, quantitative RT-PCR, immunologic detection of a human BMP-2 inducible kinase polypeptide, or measurement of enzymatic activity.

20

In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate thera-peutic agents. Selection of the appropriate agents for use in combination therapy can be made by one of ordinary skill in the art, according to conventional pharmaceutical

25   principles. The combination of therapeutic agents can act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

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Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

5   *Diagnostic methods*

Human BMP-2 inducible kinase also can be used in diagnostic assays for detecting diseases and abnormalities or susceptibility to diseases and abnormalities related to the presence of mutations in the nucleic acid sequences that encode the enzyme. For

10   example, differences can be determined between the cDNA or genomic sequence encoding BMP-2 inducible kinase in individuals afflicted with a disease and in normal individuals. If a mutation is observed in some or all of the afflicted individuals but not in normal individuals, then the mutation is likely to be the causative agent of the disease.

15

Sequence differences between a reference gene and a gene having mutations can be revealed by the direct DNA sequencing method. In addition, cloned DNA segments can be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing

20   primer can be used with a double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures using radiolabeled nucleotides or by automatic sequencing procedures using fluorescent tags.

25   Genetic testing based on DNA sequence differences can be carried out by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized, for example, by high-resolution gel electrophoresis. DNA fragments of different sequences can be distinguished on denaturing formamide gradient gels in which the

30   mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (*see, e.g.*, Myers *et*

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al., *Science 230*, 1242, 1985). Sequence changes at specific locations can also be revealed by nuclease protection assays, such as RNase and S 1 protection or the chemical cleavage method (*e.g.*, Cotton *et al.*, *Proc. Natl. Acad. Sci. USA 85*, 4397-4401, 1985). Thus, the detection of a specific DNA sequence can be performed by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes and Southern blotting of genomic DNA. In addition to direct methods such as gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

Altered levels of BMP-2 inducible kinase also can be detected in various tissues. Assays used to detect levels of the receptor polypeptides in a body sample, such as blood or a tissue biopsy, derived from a host are well known to those of skill in the art and include radioimmunoassays, competitive binding assays, Western blot analysis, and ELISA assays.

All patents and patent applications cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples, which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

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EXAMPLE 1

Detection of BMP-2 inducible kinase activity

5   The polynucleotide of SEQ ID NO: 1 is cloned in frame with glutathione S-transferase (GST) in the pGEX-4T-3 vector (Amersham Pharmacia Biotech) followed by expression in Escherichia coli. The fusion protein (GST-BMP-2 inducible kinase polypeptide) is purified by affinity chromatography using glutathione-Sepharose (Amersham Pharmacia Biotech). Kinase activity is determined

10   by incubating GST-BMP-2 inducible kinase polypeptide with myelin basic protein (Sigma) and 5 µCi of [gamma 32P]ATP in 50 mM Tris/HCl (pH 8.0), 25 mM MgCl2, 1 mM dithiothreitol, 20 µM ATP, 0.5 mM EGTA, and 10% glycerol (v/v). Following incubation at 30°C for 30 min, SDS-polyacrylamide gel electrophoresis and autoradiography are performed. It is shown that the polypeptide of SEQ ID NO:

15   2 has a BMP-2 inducible kinase activity.

EXAMPLE 2

Expression of recombinant human BMP-2 inducible kinase

20

The *Pichia pastoris* expression vector pPICZB (Invitrogen, San Diego, CA) is used to produce large quantities of recombinant human BMP-2 inducible kinase polypeptides in yeast. The BMP-2 inducible kinase-encoding DNA sequence is derived from SEQ ID NO: 1. Before insertion into vector pPICZB, the DNA

25   sequence is modified by well known methods in such a way that it contains at its 5'-end an initiation codon and at its 3'-end an enterokinase cleavage site, a His6 reporter tag and a termination codon. Moreover, at both termini recognition sequences for restriction endonucleases are added and after digestion of the multiple cloning site of pPICZ B with the corresponding restriction enzymes the modified

30   DNA sequence is ligated into pPICZB. This expression vector is designed for

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inducible expression in *Pichia pastoris*, driven by a yeast promoter. The resulting pPICZ/md-His6 vector is used to transform the yeast.

The yeast is cultivated under usual conditions in 5 liter shake flasks and the recombinantly produced protein isolated from the culture by affinity chromatography (Ni-NTA-Resin) in the presence of 8 M urea. The bound polypeptide is eluted with buffer, pH 3.5, and neutralized. Separation of the polypeptide from the His6 reporter tag is accomplished by site-specific proteolysis using enterokinase (Invitrogen, San Diego, CA) according to manufacturer's instructions. Purified human BMP-2 inducible kinase polypeptide is obtained.

EXAMPLE 3

Identification of test compounds that bind to BMP-2 inducible kinase polypeptides

Purified BMP-2 inducible kinase polypeptides comprising a glutathione-S-transferase protein and absorbed onto glutathione-derivatized wells of 96-well microtiter plates are contacted with test compounds from a small molecule library at pH 7.0 in a physiological buffer solution. Human BMP-2 inducible kinase polypeptides comprise the amino acid sequence shown in SEQ ID NO: 2. The test compounds comprise a fluorescent tag. The samples are incubated for 5 minutes to one hour. Control samples are incubated in the absence of a test compound.

The buffer solution containing the test compounds is washed from the wells. Binding of a test compound to a human BMP-2 inducible kinase polypeptide is detected by fluorescence measurements of the contents of the wells. A test compound that increases the fluorescence in a well by at least 15% relative to fluorescence of a well in which a test compound is not incubated is identified as a compound that binds to a human BMP-2 inducible kinase polypeptide.

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EXAMPLE 4

Identification of a test compound which decreases BMP-2 inducible kinase gene expression

5   A test compound is administered to a culture of human cells transfected with a BMP-2 inducible kinase expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells that have not been transfected is incubated for the same time without the test compound to provide a negative control.

10   RNA is isolated from the two cultures as described in Chirgwin *et al.*, *Biochem. 18*, 5294-99, 1979). Northern blots are prepared using 20 to 30 µg total RNA and hybridized with a ^{32}P-labeled BMP-2 inducible kinase-specific probe at 65°C in Express-hyb (CLONTECH). The probe comprises at least 11 contiguous nucleotides

15   selected from the complement of SEQ ID NO: 1. A test compound that decreases the BMP-2 inducible kinase-specific signal relative to the signal obtained in the absence of the test compound is identified as an inhibitor of BMP-2 inducible kinase gene expression.

20   ## EXAMPLE 5

Identification of a test compound which decreases BMP-2 inducible kinase activity

    A test compound is administered to a culture of human cells transfected with a BMP-
25   2 inducible kinase expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells that have not been transfected is incubated for the same time without the test compound to provide a negative control. Enzymatic activity is measured using the method of Kearns *et al.*, J Biol Chem 2001 Nov 9;276(45):42213-8. Briefly, a fusion protein can be made by cloning the kinase

30   domain of the human BMP-2 inducible in frame with glutathione S-transferase (GST) in an expression vector. The fusion protein is then expressed in *E. coli* and

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purified by affinity chromatography using glutathione-Sepharose. Kinase activity can be measured by incubating the fusion protein in the presence or absence of a test compound with the following: myelin basic protein, 5 μCi of γ-^{32}ATP in 50 mM Tris HCl, pH 8.0, 25 mM MgCl$_2$, 1 mM dithiothreitol, 20 μM ATP, 0.5 mM EGTA, and 10% glycerol (v/v). The mixture is incubated at 30°C for 30 minutes. SDS-polyacrylamide gel electrophoresis and autoradiography are then performed.

A test compound that decreases the enzymatic activity of the BMP-2 inducible kinase relative to the enzymatic activity in the absence of the test compound is identified as an inhibitor of BMP-2 inducible kinase activity.

EXAMPLE 6

Tissue-specific expression of BMP-2 inducible kinase

The qualitative expression pattern of BMP-2 inducible kinase in various tissues is determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Quantitative expression profiling

To demonstrate that BMP-2 inducible kinase is involved in cancer, expression is determined in the following tissues: adrenal gland, bone marrow, brain, cerebellum, colon, fetal brain, fetal liver, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle, small intestine, spinal cord, spleen, stomach, testis, thymus, thyroid, trachea, uterus, and peripheral blood lymphocytes. Expression in the following cancer cell lines also is determined: DU-145 (prostate), NCI-H125 (lung), HT-29 (colon), COLO-205 (colon), A-549 (lung), NCI-H460 (lung), HT-116 (colon), DLD-1 (colon), MDA-MD-231 (breast), LS174T (colon), ZF-75 (breast), MDA-MN-435 (breast), HT-1080, MCF-7 (breast), and U87. Matched pairs of malignant and normal tissue from the same patient also are tested.

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To demonstrate that BMP-2 inducible kinase is involved in the disease process of diabetes, the following whole body panel is screened to show predominant or relatively high expression: subcutaneous and mesenteric adipose tissue, adrenal gland, bone marrow, brain, colon, fetal brain, heart, hypothalamus, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid, trachea, and uterus. Human islet cells and an islet cell library also are tested. As a final step, the expression of BMP-2 inducible kinase in cells derived from normal individuals with the expression of cells derived from diabetic individuals is compared.

To demonstrate that BMP-2 inducible kinase is involved in CNS disorders, the following tissues are screened: fetal and adult brain, muscle, heart, lung, kidney, liver, thymus, testis, colon, placenta, trachea, pancreas, kidney, gastric mucosa, colon, liver, cerebellum, skin, cortex (Alzheimer's and normal), hypothalamus, cortex, amygdala, cerebellum, hippocampus, choroid, plexus, thalamus, and spinal cord.

To demonstrate that BMP-2 inducible kinase is involved in the disease process of COPD, the initial expression panel consists of RNA samples from respiratory tissues and inflammatory cells relevant to COPD: lung (adult and fetal), trachea, freshly isolated alveolar type II cells, cultured human bronchial epithelial cells, cultured small airway epithelial cells, cultured bronchial sooth muscle cells, cultured H441 cells (Clara-like), freshly isolated neutrophils and monocytes, and cultured monocytes (macrophage-like). Body map profiling also is carried out, using total RNA panels purchased from Clontech. The tissues are adrenal gland, bone marrow, brain, colon, heart, kidney, liver, lung, mammary gland, pancreas, prostate, salivary gland, skeletal muscle, small intestine, spleen, stomach, testis, thymus, trachea, thyroid, and uterus.

Quantitative expression profiling is performed by the form of quantitative PCR analysis called "kinetic analysis" firstly described in Higuchi *et al.*, *BioTechnology*

10, 413-17, 1992, and Higuchi *et al., BioTechnology 11,* 1026-30, 1993. The principle is that at any given cycle within the exponential phase of PCR, the amount of product is proportional to the initial number of template copies.

If the amplification is performed in the presence of an internally quenched fluorescent oligonucleotide (TaqMan probe) complementary to the target sequence, the probe is cleaved by the 5'-3' endonuclease activity of Taq DNA polymerase and a fluorescent dye released in the medium (Holland *et al., Proc. Natl. Acad. Sci. U.S.A. 88,* 7276-80, 1991). Because the fluorescence emission will increase in direct proportion to the amount of the specific amplified product, the exponential growth phase of PCR product can be detected and used to determine the initial template concentration (Heid *et al., Genome Res. 6,* 986-94, 1996, and Gibson *et al., Genome Res. 6,* 995-1001, 1996).

The amplification of an endogenous control can be performed to standardize the amount of sample RNA added to a reaction. In this kind of experiment, the control of choice is the 18S ribosomal RNA. Because reporter dyes with differing emission spectra are available, the target and the endogenous control can be independently quantified in the same tube if probes labeled with different dyes are used. All "real time PCR" measurements of fluorescence are made in the ABI Prism 7700.

RNA extraction and cDNA preparation. Total RNA from the tissues listed above are used for expression quantification. RNAs labeled "from autopsy" were extracted from autoptic tissues with the TRIzol reagent (Life Technologies, MD) according to the manufacturer's protocol.

Fifty µg of each RNA were treated with DNase I for 1 hour at 37°C in the following reaction mix: 0.2 U/µl RNase-free DNase I (Roche Diagnostics, Germany); 0.4 U/µl RNase inhibitor (PE Applied Biosystems, CA); 10 mM Tris-HCl pH 7.9; 10 mM MgCl$_2$; 50 mM NaCl; and 1 mM DTT.

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After incubation, RNA is extracted once with 1 volume of phenol:chloroform:-isoamyl alcohol (24:24:1) and once with chloroform, and precipitated with 1/10 volume of 3 M sodium acetate, pH5.2, and 2 volumes of ethanol.

5   Fifty µg of each RNA from the autoptic tissues are DNase treated with the DNA-free kit purchased from Ambion (Ambion, TX). After resuspension and spectro-photometric quantification, each sample is reverse transcribed with the TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA) according to the manufacturer's protocol. The final concentration of RNA in the reaction mix is
10   200 ng/µL. Reverse transcription is carried out with 2.5µM of random hexamer primers.

TaqMan quantitative analysis. Specific primers and probe are designed according to the recommendations of PE Applied Biosystems; the probe can be labeled at the 5'
15   end FAM (6-carboxy-fluorescein) and at the 3' end with TAMRA (6-carboxy-tetramethyl-rhodamine). Quantification experiments are performed on 10 ng of reverse transcribed RNA from each sample. Each determination is done in triplicate.

Total cDNA content is normalized with the simultaneous quantification (multiplex
20   PCR) of the 18S ribosomal RNA using the Pre-Developed TaqMan Assay Reagents (PDAR) Control Kit (PE Applied Biosystems, CA).

The assay reaction mix is as follows: 1X final TaqMan Universal PCR Master Mix (from 2X stock) (PE Applied Biosystems, CA); 1X PDAR control – 18S RNA (from
25   20X stock); 300 nM forward primer; 900 nM reverse primer; 200 nM probe; 10 ng cDNA; and water to 25 µl.

Each of the following steps are carried out once: pre PCR, 2 minutes at 50° C, and 10 minutes at 95°C. The following steps are carried out 40 times: denaturation, 15
30   seconds at 95°C, annealing/extension, 1 minute at 60°C.

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The experiment is performed on an ABI Prism 7700 Sequence Detector (PE Applied Biosystems, CA). At the end of the run, fluorescence data acquired during PCR are processed as described in the ABI Prism 7700 user's manual in order to achieve better background subtraction as well as signal linearity with the starting target quantity.

EXAMPLE 7

Proliferation inhibition assay: Antisense oligonucleotides suppress the growth of cancer cell lines

The cell line used for testing is the human colon cancer cell line HCT116. Cells are cultured in RPMI-1640 with 10-15% fetal calf serum at a concentration of 10,000 cells per milliliter in a volume of 0.5 ml and kept at 37°C in a 95% air/5%CO_2 atmosphere.

Phosphorothioate oligoribonucleotides are synthesized on an Applied Biosystems Model 380B DNA synthesizer using phosphoroamidite chemistry. A sequence of 24 bases complementary to the nucleotides at position 1 to 24 of SEQ ID NO: 1 is used as the test oligonucleotide. As a control, another (random) sequence is used: 5'-TCA ACT GAC TAG ATG TAC ATG GAC-3'. Following assembly and deprotection, oligonucleotides are ethanol-precipitated twice, dried, and suspended in phosphate buffered saline at the desired concentration. Purity of the oligonucleotides is tested by capillary gel electrophoresis and ion exchange HPLC. The purified oligo-nucleotides are added to the culture medium at a concentration of 10 µM once per day for seven days.

The addition of the test oligonucleotide for seven days results in significantly reduced expression of human BMP-2 inducible kinase as determined by Western blotting. This effect is not observed with the control oligonucleotide. After 3 to 7 days, the number of cells in the cultures is counted using an automatic cell counter. The

number of cells in cultures treated with the test oligonucleotide (expressed as 100%) is compared with the number of cells in cultures treated with the control oligonucleotide. The number of cells in cultures treated with the test oligonucleotide is not more than 30% of control, indicating that the inhibition of human BMP-2 inducible kinase has an anti-proliferative effect on cancer cells.

EXAMPLE 8.

In vivo testing of compounds/target validation

Acute Mechanistic Assays

Reduction in Mitogenic Plasma Hormone Levels

This non-tumor assay measures the abil$:$:$:$ of a compound to reduce either the endogenous level of a circulating hormone or the level of hormone produced in response to a biologic stimulus. Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.). At a predetermined time after administration of test compound, blood plasma is collected. Plasma is assayed for levels of the hormone of interest. If the normal circulating levels of the hormone are too low and/or variable to provide consistent results, the level of the hormone may be elevated by a pre-treatment with a biologic stimulus (i.e., LHRH may be injected i.m. into mice at a dosage of 30 ng/mouse to induce a burst of testosterone synthesis). The timing of plasma collection would be adjusted to coincide with the peak of the induced hormone response. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is p value ≤ 0.05 compared to the vehicle control group.

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Hollow Fiber Mechanism of Action Assay

Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol, these may include assays for gene expression (bDNA, PCR, or Taqman), or a specific biochemical activity (i.e., cAMP levels. Results are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at $p \leq 0.05$ as compared to the vehicle control group.

Subacute Functional *In Vivo* Assays

Reduction in Mass of Hormone Dependent Tissues

This is another on-tumor assay that measures the ability of a compound to reduce the mass of a hormone dependent tissue (i.e., seminal vesicles in males and uteri in females). Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.) according to a predetermined schedule and for a predetermined duration (i.e., 1 week). At termination of the study, animals are weighed, the target organ is excised, any fluid is expressed, and the weight of the organ is recorded. Blood plasma may also be collected. Plasma may be assayed for levels of a hormone of interest or for levels of test agent. Organ weights may be directly compared or they may be normalized for the body weight of the animal. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is p value ≤ 0.05 compared to the vehicle control group.

Hollow Fiber Proliferation Assay

Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or

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s.c. Fibers are harvested in accordance with specific readout assay protocol. Cell proliferation is determined by measuring a marker of cell number (i.e., MTT or LDH). The cell number and change in cell number from the starting inoculum are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at $p \leq 0.05$ as compared to the vehicle control group.

Anti-angiogenesis Models
Corneal Angiogenesis

Hydron pellets with or without growth factors or cells are implanted into a micro-pocket surgically created in the rodent cornea. Compound administration may be systemic or local (compound mixed with growth factors in the hydron pellet). Corneas are harvested at 7 days post implantation immediately following intracardiac infusion of colloidal carbon and are fixed in 10% formalin. Readout is qualitative scoring and/or image analysis. Qualitative scores are compared by Rank Sum test. Image analysis data is evaluated by measuring the area of neovascularization (in pixels) and group averages are compared by Student's t-test (2 tail). Significance is $p \leq 0.05$ as compared to the growth factor or cells only group.

Matrigel Angiogenesis

Matrigel, containing cells or growth factors, is injected subcutaneously. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Matrigel plugs are harvested at predetermined time point(s) and prepared for readout. Readout is an ELISA-based assay for hemoglobin concentration and/or histological examination (i.e. vessel count, special staining for endothelial surface markers: CD31, factor-8). Readouts are analyzed by Student's t-test, after the variance between groups is compared by an F-test, with significance determined at $p \leq 0.05$ as compared to the vehicle control group.

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Primary Antitumor Efficacy

Early Therapy Models
Subcutaneous Tumor

5.

Tumor cells or fragments are implanted subcutaneously on Day 0. Vehicle and/or compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule starting at a time, usually on Day 1, prior to the ability to measure the tumor burden. Body weights and tumor measurements are recorded 2-3 times weekly.

10   Mean net body and tumor weights are calculated for each data collection day. Antitumor efficacy may be initially determined by comparing the size of treated (T) and control (C) tumors on a given day by a Student's t-test, after the variance between groups is compared by an F-test, with significance determined at $p \leq 0.05$. The experiment may also be continued past the end of dosing in which case tumor

15   measurements would continue to be recorded to monitor tumor growth de:··:··. Tumor growth delays are expressed as the difference in the median time for the ':·gated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size.

20   Significance is $p \leq 0.05$.

Intraperitoneal/Intracranial Tumor Models

Tumor cells are injected intraperitoneally or intracranially on Day 0. Compounds are

25   administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule starting on Day 1. Observations of morbidity and/or mortality are recorded twice daily. Body weights are measured and recorded twice weekly. Morbidity/mortality data is expressed in terms of the median time of survival and the number of long-term survivors is indicated separately. Survival times are used to generate Kaplan-

30   Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment.

Established Disease Model

Tumor cells or fragments are implanted subcutaneously and grown to the desired size

5   for treatment to begin. Once at the predetermined size range, mice are randomized
into treatment groups. Compounds are administered p.o., i.p., i.v., i.m., or s.c.
according to a predetermined schedule. Tumor and body weights are measured and
recorded 2-3 times weekly. Mean tumor weights of all groups over days post
inoculation are graphed for comparison. An F-test is preformed to determine if the

10   variance is equal or unequal followed by a Student's t-test to compare tumor sizes in
the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as
compared to the control group. Tumor measurements may be recorded after dosing
has stopped to monitor tumor growth delay. Tumor growth delays are expressed as
the difference in the median time for the treated and control groups to attain a

15   predetermined size divided by the median time for the control group to attain that
size. Growth delays are compared by generating Kaplan-Meier curves from the times
for individual tumors to attain the evaluation size. Significance is p value ≤ 0.05
compared to the vehicle control group.

20   *Orthotopic Disease Models*
Mammary Fat Pad Assay

Tumor cells or fragments, of mammary adenocarcinoma origin, are implanted
directly into a surgically exposed and reflected mammary fat pad in rodents. The fat

25   pad is placed back in its original position and the surgical site is closed. Hormones
may also be administered to the rodents to support the growth of the tumors.
Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined
schedule. Tumor and body weights are measured and recorded 2-3 times weekly.
Mean tumor weights of all groups over days post inoculation are graphed for

30   comparison. An F-test is preformed to determine if the variance is equal or unequal
followed by a Student's t-test to compare tumor sizes in the treated and control

groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group.

Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is p value≤ 0.05 compared to the vehicle control group. In addition, this model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ, or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Intraprostatic Assay

Tumor cells or fragments, of prostatic adenocarcinoma origin, are implanted directly into a surgically exposed dorsal lobe of the prostate in rodents. The prostate is externalized through an abdominal incision so that the tumor can be implanted specifically in the dorsal lobe while verifying that the implant does not enter the seminal vesicles. The successfully inoculated prostate is replaced in the abdomen and the incisions through the abdomen and skin are closed. Hormones may also be administered to the rodents to support the growth of the tumors. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated

and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the lungs), or measuring the target organ weight (i.e., the regional lymph nodes). The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Intrabronchial Assay

Tumor cells of pulmonary origin may be implanted intrabronchially by making an incision through the skin and exposing the trachea. The trachea is pierced with the beveled end of a 25 gauge needle and the tumor cells are inoculated into the main bronchus using a flat-ended 27 gauge needle with a 90° bend. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the contralateral lung), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Intracecal Assay

Tumor cells of gastrointestinal origin may be implanted intracecally by making an
abdominal incision through the skin and externalizing the intestine. Tumor cells are
5   inoculated into the cecal wall without penetrating the lumen of the intestine using a
27 or 30 gauge needle. Compounds are administered p.o., i.p., i.v., i.m., or s.c.
according to a predetermined schedule. Body weights are measured and recorded 2-3
times weekly. At a predetermined time, the experiment is terminated and the animal
is dissected. The size of the primary tumor is measured in three dimensions using
10 either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is
preformed to determine if the variance is equal or unequal followed by a Student's t-
test to compare tumor sizes in the treated and control groups at the end of treatment.
Significance is $p \leq 0.05$ as compared to the control group. This model provides an
opportunity to increase the rate of spontaneous metastasis of this type of tumor.
15 Metastasis can be assessed at termination of the study by counting the number of
visible foci per target organ (i.e., the liver), or measuring the target organ weight. The
means of these endpoints are compared by Student's t-test after conducting an F-test,
with significance determined at $p \leq 0.05$ compared to the control group in the
experiment.

20

Secondary (Metastatic) Antitumor Efficacy

Spontaneous Metastasis

25 Tumor cells are inoculated s.c. and the tumors allowed to grow to a predetermined
range for spontaneous metastasis studies to the lung or liver. These primary tumors
are then excised. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according
to a predetermined schedule which may include the period leading up to the excision
of the primary tumor to evaluate therapies directed at inhibiting the early stages of
30 tumor metastasis. Observations of morbidity and/or mortality are recorded daily.
Body weights are measured and recorded twice weekly. Potential endpoints include

survival time, numbers of visible foci per target organ, or target organ weight. When survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment for both of these endpoints.

Forced Metastasis

Tumor cells are injected into the tail vein, portal vein, or the left ventricle of the heart in experimental (forced) lung, liver, and bone metastasis studies, respectively. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance at $p \leq 0.05$ compared to the vehicle control group in the experiment for both endpoints.

EXAMPLE 9

Diabetes: In vivo testing of compounds/target validation

5   Glucose Production

Over-production of glucose by the liver, due to an enhanced rate of gluconeogenesis, is the major cause of fasting hyperglycemia in diabetes. Overnight fasted normal rats or mice have elevated rates of gluconeogenesis as do streptozotocin-induced diabetic

10   rats or mice fed ad libitum. Rats are made diabetic with a single intravenous injection of 40 mg/kg of streptozotocin while C57BL/KsJ mice are given 40-60 mg/kg i.p. for 5 consecutive days. Blood glucose is measured from tail-tip blood and then compounds are administered via different routes (p.o., i.p., i.v., s.c.). Blood is collected at various times thereafter and glucose measured. Alternatively,

15   compounds are administered for several days, then the animals are fasted overnight, blood is collected and plasma glucose measured. Compounds that inhibit glucose production will decrease plasma glucose levels compared to the vehicle-treated control group.

20   Insulin Sensitivity

Both ob/ob and db/db mice as well as diabetic Zucker rats are hyperglycemic, hyperinsulinemic and insulin resistant. The animals are pre-bled, their glucose levels measured, and then they are grouped so that the mean glucose level is the same for

25   each group. Compounds are administered daily either q.d. or b.i.d. by different routes (p.o., i.p., s.c.) for 7-28 days. Blood is collected at various times and plasma glucose and insulin levels determined. Compounds that improve insulin sensitivity in these models will decrease both plasma glucose and insulin levels when compared to the vehicle-treated control group.

30

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Insulin Secretion

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, compounds are administered by different routes (p.o., i.p., s.c. or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4 g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1 g/kg), bled again after 15, 30, 60 and 90 minutes and plasma glucose levels determined. Compounds that increase insulin levels will decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, test compounds which regulate BMP-2 inducible kinase are administered by different routes (p.o., i.p., s.c., or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4 g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Test compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1g/kg), bled again after 15, 30, 60, and 90 minutes and plasma glucose levels determined. Test compounds that increase insulin levels will decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

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EXAMPLE 10

In vivo testing of compounds/target validation

5   Pain

Acute pain. Acute pain is measured on a hot plate mainly in rats. Two variants of hot plate testing are used: In the classical variant animals are put on a hot surface (52 to 56°C) and the latency time is measured until the animals show nocifensive behavior,

10   such as stepping or foot licking. The other variant is an increasing temperature hot plate where the experimental animals are put on a surface of neutral temperature. Subsequently this surface is slowly but constantly heated until the animals begin to lick a hind paw. The temperature which is reached when hind paw licking begins is a measure for pain threshold.

15

Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

20   *Persistent pain.* Persistent pain is measured with the formalin or capsaicin test, mainly in rats. A solution of 1 to 5% formalin or 10 to 100 μg capsaicin is injected into one hind paw of the experimental animal. After formalin or capsaicin application the animals show nocifensive reactions like flinching, licking and biting of the affected paw. The number of nocifensive reactions within a time frame of up to 90

25   minutes is a measure for intensity of pain.

Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to formalin or capsaicin administration.

30

Neuropathic pain. Neuropathic pain is induced by different variants of unilateral sciatic nerve injury mainly in rats. The operation is performed under anesthesia. The first variant of sciatic nerve injury is produced by placing loosely constrictive ligatures around the common sciatic nerve. The second variant is the tight ligation of

5   about the half of the diameter of the common sciatic nerve. In the next variant, a group of models is used in which tight ligations or transections are made of either the L5 and L6 spinal nerves, or the L% spinal nerve only. The fourth variant involves an axotomy of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact whereas the last

10   variant comprises the axotomy of only the tibial branch leaving the sural and common nerves uninjured. Control animals are treated with a sham operation.

Postoperatively, the nerve-injured animals develop a chronic mechanical allodynia, cold allodynioa, as well as a thermal hyperalgesia. Mechanical allodynia is measured

15   by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA; Electronic von Frey System, Somedic Sales AB, Hörby, Sweden). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy), or by means of a cold plate of 5 to 10°C where the nocifensive reactions of the affected

20   hind paw are counted as a measure of pain intensity. A further test for cold induced pain is the counting of nocifensive reactions, or duration of nocifensive responses after plantar administration of acetone to the affected hind limb. Chronic pain in general is assessed by registering the circadanian rhythms in activity (Surjo and Arndt, Universität zu Köln, Cologne, Germany), and by scoring differences in gait

25   (foot print patterns; FOOTPRINTS program, Klapdor et al., 1997. A low cost method to analyze footprint patterns. J. Neurosci. Methods 75, 49-54).

Compounds are tested against sham operated and vehicle treated control groups. Substance application is performed at different time points via different application

30   routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Inflammatory. Pain. Inflammatory pain is induced mainly in rats by injection of 0.75 mg carrageenan or complete Freund's adjuvant into one hind paw. The animals develop an edema with mechanical allodynia as well as thermal hyperalgesia. Mechanical allodynia is measured by means of a pressure transducer (electronic von

5   Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy, Paw thermal stimulator, G. Ozaki, University of California, USA). For edema measurement two methods are being used. In the first method, the animals are sacrificed and the affected hindpaws sectioned and weighed.

10   The second method comprises differences in paw volume by measuring water displacement in a plethysmometer (Ugo Basile, Comerio, Italy).

Compounds are tested against uninflamed as well as vehicle treated control groups. Substance application is performed at different time points via different application

15   routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Diabetic neuropathic pain. Rats treated with a single intraperitoneal injection of 50 to 80 mg/kg streptozotocin develop a profound hyperglycemia and mechanical allodynia within 1 to 3 weeks. Mechanical allodynia is measured by means of a

20   pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA).

Compounds are tested against diabetic and non-diabetic vehicle treated control groups. Substance application is performed at different time points via different

25   application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Parkinson's disease

30   *6-Hydroxydopamine (6-OH-DA) Lesion.* Degeneration of the dopaminergic ni-grostriatal and striatopallidal pathways is the central pathological event in

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Parkinson's disease. This disorder has been mimicked experimentally in rats using single/sequential unilateral stereotaxic injections of 6-OH-DA into the medium forebrain bundle (MFB).

5   Male Wistar rats (Harlan Winkelmann, Germany), weighing 200±250 g at the beginning of the experiment, are used. The rats are maintained in a temperature- and humidity-controlled environment under a 12 h light/dark cycle with free access to food and water when not in experimental sessions. The following in vivo protocols are approved by the governmental authorities. All efforts are made to minimize
10   animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

Animals are administered pargyline on the day of surgery (Sigma, St. Louis, MO, USA; 50 mg/kg i.p.) in order to inhibit metabolism of 6-OHDA by monoamine
15   oxidase and desmethylimipramine HCl (Sigma; 25 mg/kg i.p.) in order to prevent uptake of 6-OHDA by noradrenergic terminals. Thirty minutes later the rats are anesthetized with sodium pentobarbital (50 mg/kg) and placed in a stereotaxic frame. In order to lesion the DA nigrostriatal pathway 4 μl of 0.01% ascorbic acid-saline containing 8 μg of 6-OHDA HBr (Sigma) are injected into the left medial fore-brain
20   bundle at a rate of 1 μl/min (2.4 mm anterior, 1.49 mm lateral, -2.7 mm ventral to Bregma and the skull surface). The needle is left in place an additional 5 min to allow diffusion to occur.

Stepping Test. Forelimb akinesia is assessed three weeks following lesion placement
25   using a modified stepping test protocol. In brief, the animals are held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part above the surface. One paw is touching the table, and is then moved slowly sideways (5 s for 1 m), first in the forehand and then in the backhand direction. The number of adjusting steps is counted for both paws in the backhand and forehand direction of
30   movement. The sequence of testing is right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test is repeated

three times on three consecutive days, after an initial training period of three days prior to the first testing. Forehand adjusted stepping reveals no consistent differences between lesioned and healthy control animals. Analysis is therefore restricted to backhand adjusted stepping.

Balance Test. Balance adjustments following postural challenge are also measured during the stepping test sessions. The rats are held in the same position as described in the stepping test and, instead of being moved sideways, tilted by the experimenter towards the side of the paw touching the table. This maneuver results in loss of balance and the ability of the rats to regain balance by forelimb movements is scored on a scale ranging from 0 to 3. Score 0 is given for a normal forelimb placement. When the forelimb movement is delayed but recovery of postural balance detected, score 1 is given. Score 2 represents a clear, yet insufficient, forelimb reaction, as evidenced by muscle contraction, but lack of success in recovering balance, and score 3 is given for no reaction of movement. The test is repeated three times a day on each side for three consecutive days after an initial training period of three days prior to the first testing.

Staircase Test (Paw Reaching). A modified version of the staircase test is used for evaluation of paw reaching behavior three weeks following primary and secondary lesion placement. Plexiglass test boxes with a central platform and a removable staircase on each side are used. The apparatus is designed such that only the paw on the same side at each staircase can be used, thus providing a measure of independent forelimb use. For each test the animals are left in the test boxes for 15 min. The double staircase is filled with 7 x 3 chow pellets (Precision food pellets, formula: P, purified rodent diet, size 45 mg; Sandown Scientific) on each side. After each test the number of pellets eaten (successfully retrieved pellets) and the number of pellets taken (touched but dropped) for each paw and the success rate (pellets eaten/pellets taken) are counted separately. After three days of food deprivation (12 g per animal per day) the animals are tested for 11 days. Full analysis is conducted only for the last five days.

MPTP treatment.   The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes degeneration of mesencephalic dopaminergic (DAergic) neurons in rodents, non-human primates, and humans and, in so doing, reproduces many of the symptoms of Parkinson's disease. MPTP leads to a marked decrease in the levels of dopamine and its metabolites, and in the number of dopaminergic terminals in the striatum as well as severe loss of the tyrosine hydroxylase (TH)-immunoreactive cell bodies in the substantia nigra, pars compacta.

In order to obtain severe and long-lasting lesions, and to reduce mortality, animals receive single injections of MPTP, and are then tested for severity of lesion 7–10 days later. Successive MPTP injections are administered on days 1, 2 and 3. Animals receive application of 4 mg/kg MPTP hydrochloride (Sigma) in saline once daily. All injections are intraperitoneal (i.p.) and the MPTP stock solution is frozen between injections. Animals are decapitated on day 11.

Immunohistology. At the completion of behavioral experiments, all animals are anaesthetized with 3 ml thiopental (1 g/40 ml i.p., Tyrol Pharma). The mice are perfused transcardially with 0.01 M PBS (pH 7.4) for 2 min, followed by 4% paraformaldehyde (Merck) in PBS for 15 min. The brains are removed and placed in 4% paraformaldehyde for 24 h at 4°C. For dehydration they are then transferred to a 20% sucrose (Merck) solution in 0.1 M PBS at 4°C until they sink. The brains are frozen in methylbutan at -20°C for 2 min and stored at -70°C. Using a sledge microtome (mod. 3800-Frigocut, Leica), 25 μm sections are taken from the genu of the corpus callosum (AP 1.7 mm) to the hippocampus (AP 21.8 mm) and from AP 24.16 to AP 26.72. Forty-six sections are cut and stored in assorters in 0.25 M Tris buffer (pH 7.4) for immunohistochemistry.

A series of sections is processed for free-floating tyrosine hydroxylase (TH) immuno-histochemistry. Following three rinses in 0.1 M PBS, endogenous peroxidase activity is quenched for 10 min in 0.3% H_2O_2 ±PBS. After rinsing in PBS, sections

are preincubated in 10% normal bovine serum (Sigma) for 5 min as blocking agent and transferred to either primary anti-rat TH rabbit antiserum (dilution 1:2000).

Following overnight incubation at room temperature, sections for TH immuno-
5   reactivity are rinsed in PBS (2 x10 min) and incubated in biotinylated anti-rabbit immunoglobulin G raised in goat (dilution 1:200) (Vector) for 90 min, rinsed repeatedly and transferred to Vectastain ABC (Vector) solution for 1 h. 3,.3' -Diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.1 M PBS, supplemented with 0.005% H_2O_2, serves as chromogen in the subsequent visualization reaction.
10   Sections are mounted on to gelatin-coated slides, left to dry overnight, counter-stained with hematoxylin dehydrated in ascending alcohol concentrations and cleared in butylacetate. Coverslips are mounted on entellan.

Rotarod Test. We use a modification of the procedure described by Rozas and
15   Labandeira-Garcia (1997), with the CR-1 Rotamex system (Columbus Instruments, Columbus, OH) comprising an IBM-compatible personal computer, a CIO-24 data acquisition card, a control unit, and a four-lane rotarod unit. The rotarod unit consists of a rotating spindle (diameter 7.3 cm) and individual compartments for each mouse. The system software allows preprogramming of session protocols with varying
20   rotational speeds (0–80 rpm). Infrared beams are used to detect when a mouse has fallen onto the base grid beneath the rotarod. The system logs the fall as the end of the experiment for that mouse, and the total time on the rotarod, as well as the time of the fall and all the set-up parameters, are recorded. The system also allows a weak current to be passed through the base grid, to aid training.
25

Dementia

The object recognition task. The object recognition task has been designed to assess the effects of experimental manipulations on the cognitive performance of rodents. A
30   rat is placed in an open field, in which two identical objects are present. The rats inspects both objects during the first trial of the object recognition task. In a second

trial, after a retention interval of for example 24 hours, one of the two objects used in the first trial, the 'familiar' object, and a novel object are placed in the open field. The inspection time at each of the objects is registered. The basic measures in the OR task is the time spent by a rat exploring the two object the second trial. Good retention is reflected by higher exploration times towards the novel than the 'familiar' object.

Administration of the putative cognition enhancer prior to the first trial predominantly allows assessment of the effects on acquisition, and eventually on consolidation processes. Administration of the testing compound after the first trial allows to assess the effects on consolidation processes, whereas administration before the second trial allows to measure effects on retrieval processes.

The passive avoidance task. The passive avoidance task assesses memory performance in rats and mice. The inhibitory avoidance apparatus consists of a two-compartment box with a light compartment and a dark compartment. The two compartments are separated by a guillotine door that can be operated by the experimenter. A threshold of 2 cm separates the two compartments when the guillotine door is raised. When the door is open, the illumination in the dark compartment is about 2 lux. The light intensity is about 500 lux at the center of the floor of the light compartment.

Two habituation sessions, one shock session, and a retention session are given, separated by inter-session intervals of 24 hours. In the habituation sessions and the retention session the rat is allowed to explore the apparatus for 300 sec. The rat is placed in the light compartment, facing the wall opposite to the guillotine door. After an accommodation period of 15 sec. the guillotine door is opened so that all parts of the apparatus can be visited freely. Rats normally avoid brightly lit areas and will enter the dark compartment within a few seconds.

In the shock session the guillotine door between the compartments is lowered as soon as the rat has entered the dark compartment with its four paws, and a scrambled 1 mA footshock is administered for 2 sec. The rat is removed from the apparatus and put back into its home cage. The procedure during the retention session is identical to that of the habituation sessions.

The step-through latency, that is the first latency of entering the dark compartment (in sec.) during the retention session is an index of the memory performance of the animal; the longer the latency to enter the dark compartment, the better the retention is. A testing compound in given half an hour before the shock session, together with 1 mg*kg⁻¹ scopolamine. Scopolamine impairs the memory performance during the retention session 24 hours later. If the test compound increases the enter latency compared with the scopolamine-treated controls, is likely to possess cognition enhancing potential.

The Morris water escape task. The Morris water escape task measures spatial orientation learning in rodents. It is a test system that has extensively been used to investigate the effects of putative therapeutic on the cognitive functions of rats and mice. The performance of an animal is assessed in a circular water tank with an escape platform that is submerged about 1 cm below the surface of the water. The escape platform is not visible for an animal swimming in the water tank. Abundant extra-maze cues are provided by the furniture in the room, including desks, computer equipment, a second water tank, the presence of the experimenter, and by a radio on a shelf that is playing softly. .

The animals receive four trials during five daily acquisition sessions. A trial is started by placing an animal into the pool, facing the wall of the tank. Each of four starting positions in the quadrants north, east, south, and west is used once in a series of four trials; their order is randomized. The escape platform is always in the same position. A trial is terminated as soon as the animal had climbs onto the escape platform or when 90 seconds have elapsed, whichever event occurs first. The animal is allowed

to stay on the platform for 30 seconds. Then it is taken from the platform and the next trial is started. If an animal did not find the platform within 90 seconds it is put on the platform by the experimenter and is allowed to stay there for 30 seconds. After the fourth trial of the fifth daily session, an additional trial is given as a probe trial:

5 the platform is removed, and the time the animal spends in the four quadrants is measured for 30 or 60 seconds. In the probe trial, all animals start from the same start position, opposite to the quadrant where the escape platform had been positioned during acquisition.

10 Four different measures are taken to evaluate the performance of an animal during acquisition training: escape latency, traveled distance, distance to platform, and swimming speed. The following measures are evaluated for the probe trial: time (s) in quadrants and traveled distance (cm) in the four quadrants. The probe trial provides additional information about how well an animal learned the position of the

15 escape platform. If an animal spends more time and swims a longer distance in the quadrant where the platform had been positioned during the acquisition sessions than in any other quadrant, one concludes that the platform position has been learned well.

In order to assess the effects of putative cognition enhancing compounds, rats or mice

20 with specific brain lesions which impair cognitive functions, or animals treated with compounds such as scopolamine or MK-801, which interfere with normal learning, or aged animals which suffer from cognitive deficits, are used.

The T-maze spontaneous alternation task. The T-maze spontaneous alternation task

25 (TeMCAT) assesses the spatial memory performance in mice. The start arm and the two goal arms of the T-maze are provided with guillotine doors which can be operated manually by the experimenter. A mouse is put into the start arm at the beginning of training. The guillotine door is closed. In the first trial, the 'forced trial', either the left or right goal arm is blocked by lowering the guillotine door. After the

30 mouse has been released from the start arm, it will negotiate the maze, eventually enter the open goal arm, and return to the start position, where it will be confined for

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5 seconds, by lowering the guillotine door. Then, the animal can choose freely between the left and right goal arm (all guillotine-doors opened) during 14 'free choice' trials. As soon a the mouse has entered one goal arm, the other one is closed. The mouse eventually returns to the start arm and is free to visit whichever go alarm it wants after having been confined to the start arm for 5 seconds. After completion of 14 free choice trials in one session, the animal is removed from the maze. During training, the animal is never handled.

The percent alternations out of 14 trials is calculated. This percentage and the total time needed to complete the first forced trial and the subsequent 14 free choice trials (in s) is analyzed. Cognitive deficits are usually induced by an injection of scopolamine, 30 min before the start of the training session. Scopolamine reduced the per-cent alternations to chance level, or below. A cognition enhancer, which is always administered before the training session, will at least partially, antagonize the scopolamine-induced reduction in the spontaneous alternation rate.

EXAMPLE 11

Identification of test compound efficacy in an animal model of COPD

A/J mice are exposed to the smoke from 2 unfiltered cigarettes per day for 6 days per week for 14 weeks. Non-smoking, age-matched animals are used as controls. Animals are orally dosed with test compound or vehicle 1 hour before and 7 hours after smoke exposure. This twice-daily dosing regime is continued throughout the smoke exposure period. On day 7 of the weekly exposure, animals are given only 1 dose of test compound and are not exposed to cigarette smoke.

After the smoke exposure period, the mice are killed, their lungs inflated with phosphate-buffered formalin via their trachea, and then the lungs and heart are removed *en bloc* and fixed at 4°C for 48 hours. The lungs are then prepared for paraffin wax sectioning, and 4 mm sections are cut and mounted on glass slides.

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Sections are then stained with haematoxylin and eosin. Morphometric analysis of lung sections is done by calculation of the Linear Mean Intercept (LMI) parameter using a semi-automated computer image analysis system. Each slide (1 per mouse) contains several sections originating from multiple lobes. Twelve non-overlapping areas (each area covering 1.53 x 10-3 cm2) are randomly selected for LMI analysis. The 12 areas cover a minimum of two lobes per slide. Non-parenchymal components (airways, blood vessels) are excluded from the analysis to prevent artifactual error. The mean intercept length is calculated for each mouse. Development of emphysema is seen as an increase in LMI.

LMI data are expressed as the median and statistical comparisons are done using the non-parametric Mann-Witney U-test. A 'p' value of <=0.05 is considered to be statistically significant. The potency of a test compound is evaluated by comparison of the tobacco smoke induced increase in LMI in animals dosed with either the test compound ... just the vehicle used for administration of the compound.

EXAMPLE 12

Identification of test compound efficacy in an in vitro functional test relevant to COPD

The potency of test compounds is evaluated by measuring the inhibition of elastolysis induced by human alveolar macrophages. The cells are isolated from bronchoalveolar lavage samples taken from non-smokers, disease-free smokers, and smokers with COPD. Macrophage suspensions are added to test wells coated with tritiated elastin and incubated at 37°C for 3h to allow adherence of the cells. The wells are then carefully washed to remove non-adherent cells and fresh medium is added to each well. The cells are incubated at 37°C for up to 72 hours in the presence or absence of test compound. Every 24 hours the medium in each well is removed for analysis and replaced by fresh medium. Radioactivity released into the medium is measured by liquid scintillation counting and the rate of elastin

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degradation is calculated. The potency of a test compound is evaluated by comparing the rate of elastolysis measured with cells incubated in the presence or absence of the compound.

5   **EXAMPLE 13**

In vivo target validation

Effects on plasma cholesterol levels including HDL cholesterol are typically assessed
10   in humanized apo-AI transgenic mice. Modulation of human target proteins can be determined in corresponding transgenic mice (e.g., CETP transgenic mice). Triglyceride lowering is usually evaluated in ob/ob mice or Zucker rats. Animals are fed with normal diets or modified diets (e.g., enriched by 0.5 % cholesterol 20% coconut oil). Standard protocols consist of oral applications once daily for 7 to
15   10 days at doses ranging from 0,1 to 100 mg/kg. The compounds are dissolved (*e.g.,* in Solutol/Ethanol/saline mixtures) and applied by oral gavage or intravenous injection. Before and at the end of the application period, blood samples are typically drawn by retroorbital punctuation. Plasma cholesterol and triglyceride levels are determined with standardized clinical diagnostic kits (e.g., INFINITY™ cholesterol
20   reagent and INFINITY™ triglyceride reagent; Sigma, St. Louis). HDL cholesterol is determined after phosphotungstic acid precipitation of non-HDL lipoproteins or FPLC gel filtration with post-column derivatization of cholesterol using the reagents mentioned above. Plasma levels of human apolipoprotein-AI in relevant humanized transgenic mice are measured by immunoturbidimetry (Sigma).

25

Long-term anti-atherosclerotic potency of drug candidates are evaluated in Apo E-knockout mice. Therefore, animals are fed a standard chow diet (4,5 % fat) or a Western diet (20 % fat) containing 1 to 100 mg/kg of the respective compounds for 3 to 5 month. Arterial lesions are quantified in serial cryosections of the proximal aorta
30   by staining with Oil Red O and counterstaining with hematoxylin. Lesion area size is determined using an digital imaging system.

EXAMPLE 14

In vivo testing of cardiovascular effects of test compounds

5

Hemodynamics in anesthetized rats

Male Wistar rats weighing 300-350 g (Harlan Winkelmann, Borchen, Germany) are

anesthetized with thiopental "Nycomed" (Nycomed, Munich, Germany) 100 mg kg-1

10   i.p. A tracheotomy is performed, and catheters are inserted into the femoral artery for

blood pressure and heart rate measurements (Gould pressure transducer and recorder,

model RS 3400) and into the femoral vein for substance administration. The animals

are ventilated with room air and their body temperature is controlled.   Test

compounds are administered orally or intravenously.

15

Hemodynamics in conscious SHR

Female conscious SHR (Moellegaard/Denmark, 220 – 290 g) are equipped with

implantable radiotelemetry, and a data acquisition system (Data Sciences, St. Paul,

20   MN, USA), comprising a chronically implantable transducer/transmitter unit

equipped with a fluid-filled catheter is used. The transmitter is implanted into the

peritoneal cavity, and the sensing catheter is inserted into the descending aorta.

Single administration of test compounds is performed as a solution in Transcutol®/

25   Cremophor®/ H2O (10/20/70 = v/v/v) given orally by gavage. The animals of

control groups only receive the vehicle. Before treatment, mean blood pressure and

heart rate of treated and untreated control groups are measured.

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Hemodynamics in anesthetized dogs

Studies are performed on anesthetized dogs of either sex (body weight between 20-30 kg). Anesthesia is initiated by slow intravenous injection of 25 mg kg-1 sodium
5   thiopental (Trapanal®, Byk Gulden, Konstanz, Germany). The anesthesia is continued and maintained throughout the experiment by continuous infusion of 0.04 mg kg-1 h-1 fentanyl (Fentanyl®, Janssen, Neuss, Germany) and 0.25 mg kg-1 h□1 droperidol (DihydrobenzperidolR, Janssen, Neuss, Germany). During this anaesthesia, heart rate is as low as 35-40 bpm due to increased vagal tone. Therefore,
10   a parasympathetic blockade is achieved by intermittent injections of atropine (0.1 mg per animal) (AtropinsulfatR, Eifelfango, Bad Neuenahr, Germany). After intubation the animals are artificially ventilated at constant volume (EngströmR 300, Engström, Sweden) with room air enriched with 30% oxygen to maintain an end-tidal CO_2 concentration of about 5% (NormocapR, Datex, Finland).

15

The following catheters are implanted for measurement of cardiovascular parameters: a tip catheter for recording of left ventricular pressure is inserted into the ventricle via the carotid artery (PC350, Millar Instruments, Houston, TX, USA), a hollow catheter is inserted into the femoral artery and connected to a strain gauge (type 4-327-1,
20   Telos Medical, Upland, CA, USA for recording of arterial blood pressure, two venous catheters are inserted into either femoral vein and one additional catheter into a forearm vein for application of the anaesthetic and drugs, respectively, and an oxymetry catheter for recording of oxygen saturation is inserted into the coronary sinus via the jugular vein (Schwarzer IVH4, München, Germany).

25

After a left-sided thoracotomy the ramus circumflexus of the left coronary artery (LCX) is freed from connective tissue, and an electromagnetic flow probe (Gould Statham, Oxnard, CA, USA) is applied for measurement of coronary blood flow. Arterial blood pressure, electrocardiogram (lead II), left ventricular pressure, first
30   derivative of left ventricular pressure (dP/dt), heart rate, coronary blood flow, and oxygen saturation in the coronary sinus are continuously recorded on a pen recorder

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(Brush, Gould, Cleveland, OH, USA). The maximum of dP/dt is used as measure of left ventricular contractility (dP/dtmax). After completion of the instrumentation, an interval of 60 min is allowed for stabilization before the test compound is intravenously applied as bolus injections. Care is taken that all measured cardiovascular parameters have returned to control level before injection of the next dose. Each dose of the test compound is tested at least three times in different animals. The order of injection of the different doses is randomized in each animal.

EXAMPLE 15

Expression profiling

Total cellular RNA was isolated from cells by one of two standard methods: 1) guanidine isothiocyanate/cesium chloride density gradient centrifugation [Kellogg *et al.* (1990)]; or with the Tri-Reagent protocol according to the manufacturer's specifications (Molecular Research Center, Inc., Cincinnati, Ohio). Total RNA prepared by the Tri-reagent protocol was treated with DNAse I to remove genomic DNA contamination.

For relative quantitation of the mRNA distribution, total RNA from each cell or tissue source was first reverse transcribed. Eighty-five μg of total RNA was reverse transcribed using 1 μmole random hexamer primers, 0.5 mM each of dATP, dCTP, dGTP and dTTP (Qiagen, Hilden, Germany) and 3000 U RnaseQut (Invitrogen, Groningen, Netherlands) in a final volume of 680 μl. The first strand synthesis buffer and Omniscript reverse transcriptase (2 u/μl) were obtained from (Qiagen, Hilden, Germany). The reaction was incubated at 37°C for 90 minutes and cooled on ice. The volume was adjusted to 6800 μl with water, yielding a final concentration of 12.5 ng/μl of starting RNA.

For relative quantitation of the distribution of mRNA in cells and tissues the Perkin Elmer ABI Prism R™ 7700 Sequence Detection system or Biorad iCycler was used

according to the manufacturer's specifications and protocols. PCR reactions were set up to quantitate expression of the test gene and the housekeeping genes HPRT (hypoxanthine phosphoribosyltransferase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ß -actin, and others. Forward and reverse primers and probes were designed using the Perkin Elmer ABI Primer Express™ software and were synthesized by TibMolBiol (Berlin, Germany). The forward primer sequence was: Primer1 gcaaggttgcatcagtgtaaga. The reverse primer sequence was Primer2 tcccaccatcattcaacaaa. Probe1 tccaataattcaccgggatctgaagg, labeled with FAM (carboxyfluorescein succinimidyl ester) as the reporter dye and TAMRA (carboxy-tetramethylrhodamine) as the quencher, was used as a probe. The following reagents were prepared in a total of 25 µl: 1x TaqMan buffer A, 5.5 mM MgCl$_2$, 200 nM of dATP, dCTP, dGTP, and dUTP, 0.025 U/µl AmpliTaq Gold™, 0.01 U/µl AmpErase, and Probe1 tccaataattcaccgggatctgaagg, forward and reverse primers each at 200 nM, 200 nM, FAM/TAMRA-labeled probe, and 5 µl of template cDNA. Thermal cycling parameters were 2 min at 50°C, followed by 10 min at 95°C, followed by 40 cycles of melting at 95° C for 15 sec and annealing/extending at 60°C for 1 min.

Calculation of corrected CT values

The CT (threshold cycle) value is calculated as described in the "Quantitative determination of nucleic acids" section. The CF-value (factor for threshold cycle correction) is calculated as follows:

1.   PCR reactions were set up to quantitate the housekeeping genes (HKG) for each cDNA sample.

2.   CT$_{HKG}$-values (threshold cycle for housekeeping gene) were calculated as described in the "Quantitative determination of nucleic acids" section.

3.   CT$_{HKG}$-mean values (CT mean value of all HKG tested on one cDNAs) of all HKG for each cDNA are calculated (n = number of HKG):

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$$CT_{HKG-n}\text{-mean value} = (CT_{HKG1}\text{-value} + CT_{HKG2}\text{-value} + \ldots + CT_{HKG-n}\text{-value}) / n$$

4. CT_{pannel} mean value (CT mean value of all HKG in all tested cDNAs) =

$$(CT_{HKG1}\text{-mean value} + CT_{HKG2}\text{-mean value} + \ldots + CT_{HKG-y}\text{-mean value}) / y$$
(y = number of cDNAs)

5. CF_{cDNA-n} (correction factor for cDNA n) = CT_{pannel}-mean value - CT_{HKG-n}-mean value

6. CT_{cDNA-n} (CT value of the tested gene for the cDNA n) + CF_{cDNA-n} (correction factor for cDNA n) = $CT_{cor-cDNA-n}$ (corrected CT value for a gene on cDNA n)

Calculation of relative expression

Definition: highest $CT_{cor-cDNA-n} \neq 40$ is defined as $CT_{cor-cDNA}$ [high]
Relative Expression = $2^{(CT_{cor-cDNA[high]} - CT_{cor-cDNA-n})}$

The following tissues were tested: rectum, occipital lobe, parietal lobe, temporal lobe, skin, hippocampus, Alzheimer cerebral cortex, spinal cord, fetal kidney, thalamus, ileum, spleen, cerebral peduncles, kidney tumor, ovary, esophagus tumor, testis, fetal liver, substantia nigra, heart atrium (right), fetal heart, vermis cerebelli, precentral gyrus, Alzheimer brain frontal lobe, fetal lung, Alzheimer brain, breast, HUVEC cells, spleen liver cirrhosis, cerebral cortex, liver tumor, heart atrium (left), uterus tumor, cerebellum (left), bone marrow, HEK 293 cells, thymus, corpus callosum, kidney, fetal lung fibroblast IMR-90 cells, Jurkat (T-cells), cord blood CD71+ cells, ovary tumor, thyroid tumor, cerebellum (right), colon, interventricular septum, bone marrow stromal cells, heart, tonsilla cerebelli, neuroblastoma IMR32 cells, placenta, liver, breast tumor, thyroid, HEK CNS + APP, brain, neuroblastoma SH5Y cells, lung tumor, mammary gland, neuroblastoma SK-N-MC cells,

pericardium, fetal brain, frontal lobe, pons, ileum tumor, uterus, ileum tumor, liver cirrhosis, MDA MB 231 cells (breast tumor), retina, pancreas liver cirrhosis, colon tumor, stomach tumor, bladder, glial tumor H4 cells, stomach, HEP G2 cells, skeletal muscle, bone marrow CD71+ cells, leukocytes (peripheral blood), prostate, lymph node, bone marrow CD34+ cells, lung right lower lobe, trachea, HEK CNS, coronary artery smooth muscle primary cells, small intestine, heart ventricle (left), glial tumor H4 cells + APP, HeLa cells (cervix tumor), lung COPD, adrenal gland, pancreas, lung right upper lobe, prostate BPH, salivary gland, ileum chronic inflammation, erythrocytes, lung right mid lobe, ureter, corpus cavernosum, dorsal root ganglia, penis, fetal aorta, bone marrow CD33+ cells, postcentral gyrus, thrombocytes, cerebellum, esophagus, aorta sclerotic, bone marrow CD15+ cells, artery, aorta, cerebral meninges, adipose, coronary artery, coronary artery sclerotic, cervix, vein, lung.

The results are shown in Table 1.

Table 1

Tissue	Relative Expression
rectum	2180
occipital lobe	1652
parietal lobe	1585
temporal lobe	1552
skin	1468
hippocampus	1468
Alzheimer cerebral cortex	1468
spinal cord	1184
fetal kidney	1105
thalamus	1003
ileum	917

Tissue	Relative Expression
spleen	843
cerebral peduncles	704
kidney tumor	671
ovary	635
esophagus tumor	635
testis	596
fetal liver	541
substantia nigra	534
heart atrium (right)	534
fetal heart	516
vermis cerebelli	512
precentral gyrus	505
Alzheimer brain frontal lobe	471
fetal lung	461
Alzheimer brain	458
breast	434
HUVEC cells	391
spleen liver cirrhosis	360
cerebral cortex	350
liver tumor	347
heart atrium (left)	329
uterus tumor	320
cerebellum (left)	317
bone marrow	315
HEK 293 cells	313
thymus	309
corpus callosum	302
kidney	284
fetal lung fibroblast IMR-90 cells	282

Tissue	Relative Expression
Jurkat (T-cells)	278
cord blood CD71+ cells	276
ovary tumor	276
thyroid tumor	272
cerebellum (right)	267
colon	265
interventricular septum	265
bone marrow stromal cells	251
heart	244
tonsilla cerebelli	242
neuroblastoma IMR32 cells	239
placenta	239
liver	232
breast tumor	231
thyroid	221
HEK CNS + APP	205
brain	191
neuroblastoma SH5Y cells	189
lung tumor	184
mammary gland	184
neuroblastoma SK-N-MC cells	176
pericardium	175
fetal brain	174
frontal lobe	165
pons	162
ileum tumor	160
uterus	160
ileum tumor	160
liver cirrhosis	154

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Tissue	Relative Expression
MDA MB 231 cells (breast tumor)	152
retina	151
pancreas liver cirrhosis	135
colon tumor	129
stomach tumor	129
bladder	128
glial tumor H4 cells	123
stomach	123
HEP G2 cells	119
skeletal muscle	118
bone marrow CD71+ cells	107
leukocytes (peripheral blood)	100
prostate	96
lymph node	96
bone marrow CD34+ cells	89
lung right lower lobe	87
trachea	86
HEK CNS	82
coronary artery smooth muscle primary cells	81
small intestine	76
heart ventricle (left)	75
glial tumor H4 cells + APP	75
HeLa cells (cervix tumor)	72
lung COPD	61
adrenal gland	58
pancreas	58
lung right upper lobe	41
prostate BPH	41
salivary gland	41

Tissue	Relative Expression
ileum chronic inflammation	40
erythrocytes	37
lung right mid lobe	32
ureter	32
corpus cavernosum	32
dorsal root ganglia	27
penis	24
fetal aorta	23
bone marrow CD33+ cells	21
postcentral gyrus	20
thrombocytes	14
cerebellum	12
esophagus	11
aorta sclerotic	10
bone marrow CD15+ cells	8
artery	5
aorta	5
cerebral meninges	4
adipose	4
coronary artery	4
coronary artery sclerotic	3
cervix	2
vein	2
lung	1

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